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Isolation of SFRS3 gene and its differential expression during metamorphosis involving eye migration of Japanese flounder *Paralichthys olivaceus*

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Abstract

Both eyes of flatfishes are located on one side of the body due to asymmetrical eye migration. The molecular mechanisms underlying such asymmetry is poorly understood. As an initial step, we have adopted suppression subtractive hybridization for the identification of upregulated genes during metamorphosis involving eye migration in Japanese flounder, *Paralichthys olicaceus*. One of the upregulated genes was identified as the splicing factor arginine/serine rich-3 (SFRS3). Sequence analysis of SFRS3 revealed that it encodes a protein of 168 amino acids containing the typical eukaryotic RNA recognition motif (RRM) and an arginine/serine-rich region. The overall amino acid sequences of the Japanese flounder SFRS3 was highly conserved with that of other organisms. The expression of flounder SFRS3 gene increased sharply from the beginning of metamorphosis and reached a high level of expression at stage H of metamorphosis 43 days after hatching. The SFRS3 gene upregulation was mainly limited to the head region, particularly in the rapidly proliferative tissues, the lateral ethmoid and "skin thickness" on blind side, which are thought as two proliferative tissues to push the eye movement. In spite of the upregulated expression of SFRS3 during metamorphosis, its role in metamorphosis involving eye migration requires further studies.

Keywords: SFRS3; Fish; Flounder; Metamorphosis; Eye; Development

1. Introduction

The asymmetry of flatfishes is one of the most striking body forms among vertebrates with both eyes being located on only one side (the ocular side) of the body. Migration of eye occurs at the beginning of the asymmetrical development. It was proposed that thyroid hormone may regulate eye migration in the same way as tail regression in tadpole metamorphosis [1,2]. However, the thyroid hormone receptor was found to be universally expressed during flatfish metamorphosis, providing no evidence for its direct involvement in eye migration [3].

Recently, it has been revealed that the asymmetrical orientation of the internal organs is controlled by the molecular pathways, which establish the embryonic L/R axis and precede the organogenesis [4]. All vertebrates examined to date have a common signaling molecule related to Nodal [5]. It is expressed in the left lateral plate mesoderm and induces left-side specific expression of a bicoid-type homeobox gene pitx2, which is also conserved among vertebrates as characterized in mouse, chicken, frog, and zebrafish [6–11]. Using a reversed clonal line of Japanese flounder (*rev*), Hashimoto et al. [12] found that bilateral pitx2 expression in *rev* populations led to randomization of visceral organs, but reversal of the sidedness of the orientation of the visceral organs was not always accompanied by reversal of the direction of metamorphic eye-migration, suggesting that different mechanisms were

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involved downstream of the *rev* locus in directing these two phases of asymmetric morphogenesis in the Japanese flounder.

The objective of this study was to isolate differentially expressed genes during metamorphosis involving eye migration in Japanese flounder, *Paralichthys olivaceus*. A PCR-based cDNA subtraction technique, suppression subtraction hybridization (SSH) [13,14], was adopted in this study for the identification of genes that are upregulated during the period of eye migration. An alternative splicing factor, SFRS3 (previously named Srp20 or X16), a member of SR family of serine- and arginine-rich RNA-binding proteins, was identified as one of the differentially expressed genes during eye migration of Japanese flounder. SR proteins are thought to be essential splicing factors [15–19]. In addition, SR proteins were shown to be capable of influencing the selection of alternative splice sites in a concentration-dependent manner [20–23]. Here, we report the identification, structural analysis, and expression of SFRS3 gene during the course of metamorphosis involving eye migration.

2. Materials and methods

2.1. Animals

Japanese flounder larvae at 17 days post-hatching (dph) were obtained from the Beidaihe Central Experiment Station, Chinese Academy of Fisheries Sciences, Hebei, China, and transported to the laboratory of College of Aquatic Life Science and Technology, Shanghai Fisheries University, Shanghai. The flounder larvae were stocked in an 80-l plastic tank at a density of 7000 larvae per tank at 20 °C. The larvae were fed on the newly hatched *Artemia nauplii*. The metamorphic stages of the Japanese flounder was defined as the following: Pre-metamorphosis, the stage prior to the start of eye migration; Stage E, the right eye just start to migrate; Stage G, the right eye became visible from the ocular side; Stage H, the right eye located on the dorsal margin [2,24,25]. Under this definition, larvae of 17 dph, 23 dph, 33 dph, 43 dph represented Pre-metamorphosis, Stage E, Stage G, and Stage H, respectively.

2.2. Cloning and sequencing

To avoid contamination from DNA of the prey, head tissues were isolated from the flounder larvae, cleaned with physiological saline, and immediately used for the extraction of total RNA. Total RNA was extracted by using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Suppression subtractive hybridization was performed between 17 dph larvae (Pre-metamorphosis stage) and 23 dph larvae (stage E) using a PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. In the forward subtraction, cDNA from 23 dph larvae was used as the tester and 17 dph as the driver, whereas in the reverse subtraction, cDNA from 17 dph larvae was used as the tester and 23 dph as the driver.

The subtracted pool of cDNA fragments of forward and reverse subtraction reactions were then inserted into the T/A cloning vector pGEM-T (Promega, Madison, WI). Transformants carrying subtracted cDNA fragments were isolated using color selection agar plates containing IPTG and X-gal. The picked clones containing putative differentially expressed genes were sequenced using dideoxy chain termination method [26].

2.3. cDNA library construction and isolation of complete cDNA

Initial sequencing analysis indicated the presence of a 261-bp fragment with similarity to SFRS3 gene, but the insert contained only its partial cDNA. In order to obtain complete cDNA for the SFRS3 gene, a cDNA library was constructed using E stage flounder larvae head mRNA. The cDNAs were cloned into cloning vector λTripIEx2 (Clontech) according to manufacturer's recommendations. The SFRS3 cDNA was then obtained by using vector-anchored PCR [27]. Briefly, The upstream portion of the cDNA was amplified by using a primer in the cloning vector and a gene-specific primer that is complementary to the RNA sequences, and then the full cDNA was obtained by using upstream gene-specific sequences plus a primer designed from the vector sequences downstream of the cDNA. The following primers were used for the amplification of the full-length cDNA: SFRS3-A: 5′-ACGCGTAGTTTACAGCCACACATGTT-3′; λTripIEx2-A: 5′-CTCGGGAAGCGCGCCATTGTGTTGGT-3′; SFRS3-B: 5′-AGACAAGTGAAGCT-AGCATCAGCAG-CAG-3′; λTripIEx2-B: 5′-ATACGACTCACTATAGGGC-GAATTGGCC-3′.

2.4. Reverse transcription-polymerase chain reaction

Total RNA was used for reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed using Quantum RNA™ Universal 18S Internal Standard Kit (Ambion, Austin, TX) as described in the protocol provided by the manufacture. The RT-PCR reactions contained the following: total RNA (1 μg), 5 μmole random primers, and 40 mM dNTPs in nuclease-free water. The reaction mix was heated to 85 °C for 3 min, then cooled on ice for 1 min. cDNA synthesis was initiated by adding into the reaction mix 100 U of recombinant Moloney-murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA). The RT reaction was incubated at 42 °C for 60 min. The SFRS3-specific primers were made from the full-length cDNA sequences. The sense primer reads 5′-TAAGAAGTGTTTGGGTAGCCAGGAATCCAC-3′ and the antisense primer reads 5′-ACGCGTAGTTTACAGCCA-CACATGTTTCTC-3′. PCR was performed using a Perkins

Elmer thermal cycler 600. RT-PCR was performed using the following cycling profiles: incubate at 95 °C for 5 min before cycling, followed by denaturation for 1 min at 95 °C, annealing for 45 s at 68 °C, and elongation for 1 min at 72 °C. In order to achieve semi-quantitative RT-PCR, preliminary experiments were conducted with variable cycles at 20–30 cycles to determine the appropriate levels of amplification such that the PCR reaction were still within the linear phase. A total of 27 cycles provided with reproducible results in a semi-quantitative manner. The standard primer and competimer for 18S internal control was set at 2:8. The PCR products were analyzed on a 2% agarose gel. The band density was assessed with a software package Smartscape. (Furi Co., Shanghai, China). Three repeats of RT-PCR reactions were conducted to provide a statistical estimate of relative expression.

2.5. Whole-mount in situ hybridization

For in situ hybridization, an antisense probe and a sense probe (used as a control) for flounder SFRS3 was generated by in vitro transcription. A plasmid (pGEM-T vector) containing a segment of the flounder SFRS3 cDNA (656 bp from the start of the cDNA sequence) was linearized by restriction endonuclease digestion using *Sal*I or *Nco*I, respectively. The linear plasmid was used as template for in vitro transcription using T7 or SP6 RNA polymerase, respectively, in the presence of digoxigenin-labeled uridine triphosphate (UTP; Boehringer Mannheim, Mannheim, Germany). Five larvae from each stage were first rehydrated with series of solution with different ratio of methanol and PBS (80%, 60%, 40%, 20%) and finally with 100% PBS for 20 min each. The larvae were then treated with 0.1 M PBS containing 100 mM glycine for 30 min to eliminate paraformaldehyde. Endogenous AP activity was quenched with 0.2 M HCl for 30 min, followed by two 20-min washes in PBS. The larvae specimen treated in 1 M NaOH solution containing 3% H_2O_2 under bright light for 15 min to get rid of the pigmentation in the larvae specimen, followed by two rinses in PBS for 15 min each. Larvae were permeabilized by incubation in 0.3% Triton X-100 in PBS for 1 h, followed by two rinses in PBS for 20 min. Proteinase K (100 μg/ml) was used to digest larvae for 60 min to further premeabilize the specimen, followed by 4% PFA refix and two rinses in PBS for 20 min each. Larvae were then incubated for 4 h at 45 °C in prehybridization buffer (4× SSC with 50% formamide), then hybridized with the labeled probe for 16 h at 45 °C. The hybridization buffer consisted of 50% formamide, 1% blocking reagent (Boehringer-Mannheim), 4× SSC, 10 mM DTT and 1 mg/ml yeast t-RNA, and the labeled probe. After hybridization, the larvae were washed through a graded series of SSC (4×, 3.5×, 3×, and 2.5×) containing 50% formamide at 45 °C each for 20 min, The specimens were further washed in 2× SSC at 45 °C for

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agacaagtgaagctagcatcagcagcagcagcagtagcagaacacggcggcgtgagatct    60
gcggccaacagtgtcatttagctgtagctaccgaaaggttcatcagattatttgaagaag   120
aggacttggtttggtaaaATGGGAGATCCCGCTCTAACCAGAGACTGTCCCCTTGACTGC   180
                     M  G  D  P  A  L  T  R  D  C  P  L  D  C     14
AAGGTTTATGTCGGAAATCTGGGAAACAATGGAAACAAGACCGAATTAGAAAGGGCTTTT   240
 K  V  Y  V  G  N  L  G  N  N  G  N  K  T  E  L  E  R  A  F     34
GGGTACTATGGTCCTTTAAGAAGTGTTTGGGTAGCCAGGAATCCACCAGGTTTTGCTTTT   300
 G  Y  Y  G  P  L  R  S  V  W  V  A  R  N  P  P  G  F  A  F     54
GTAGAGTTTGAAGATCCCAGAGATGCATCTGACGCTGTTCGAGAACTGGATGGGAGAAAC   360
 V  E  F  E  D  P  R  D  A  S  D  A  V  R  E  L  D  G  R  N     74
ATGTGTGGCTGTAAACTACGCGTCGAGTTGTCCACTGGGGAAAAGCGCTCTAGGAGCCGT   420
 M  C  G  C  K  L  R  V  E  L  S  T  G  E  K  R  S  R  S  R     94
GGCCCTCCCCCATCCTGGGGTAGACGCCCCCGTGATGATTTAAGACGACGTAGTCCTCCA   480
 G  P  P  P  S  W  G  R  R  P  R  D  D  L  R  R  R  S  P  P    114
GTCAGACGCAGATCCCCGAAGAGGAGGAGCCTCAGCCGCAGTCGCAGCAGGTCTGTTTCA   540
 V  R  R  R  S  P  K  R  R  S  L  S  R  S  R  S  R  S  V  S    134
AGAGACAGACATAGATTTCGTTCTCTTTCCAGAGATAAGAATCGTAAGCACTCAAGATCC   600
 R  D  R  H  R  F  R  S  L  S  R  D  K  N  R  K  H  S  R  S    154
ATCTCACGGTCAAGGAGTACTTCCCAGTCTAACGAGAGGAAATGAgaacttcagaggatt   660
 I  S  R  S  R  S  T  S  Q  S  N  E  R  K  *                   168
cgtctgcaggatcctgctatggttgtggaaatacaaagtttttatagtcacgtttttctt   720
tttagattttgtttgtcagcttgaagcagtttaaggttagcttgttacatttggtgtaat   780
ggcagtctgttaaattgacaacaaaaacatctttctttttttggtattgaattcactgttt   840
acatgaatcaaatttttcttaccctgcatgtcaagaaattaattactggattttgcttta   900
agtttctatcctgttacttttttaactgcctaactgaattaaactacatgtgatgctttta   960
tttgtttttgttttctttttctcggctgtcctttaaattacaattggtttctgtaataat  1020
gcatcaccagtcaaaggagcatacttttttaccagtgacaaaaacagactcttaactagtg  1080
gtgtaaatactggggctgtaatatgcaggttattcttgaaaactaaaaaaatccgctgtg  1140
attctttttttcttacttatgtcacttttgcagaagcattgactcttattatttgcttgat  1200
tctctgaagttctgacaatatgtggtgatgcagtcaaacttcttgttcaataaatgtttt  1260
ccaaggtaaaaaaaaaaaaaaaaaaaaa                                   1288
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Fig. 1. Full-length cDNA and deduced amino acid sequence of flounder SFRS3. Coding regions are in upper case while the 5′ and 3′ untranslated regions are in lower case. The asterisk stands for the termination of the protein. Polyadenylation signal sequence aataaa is bold and underlined. The RNA recognition motif (RRM) is italic.

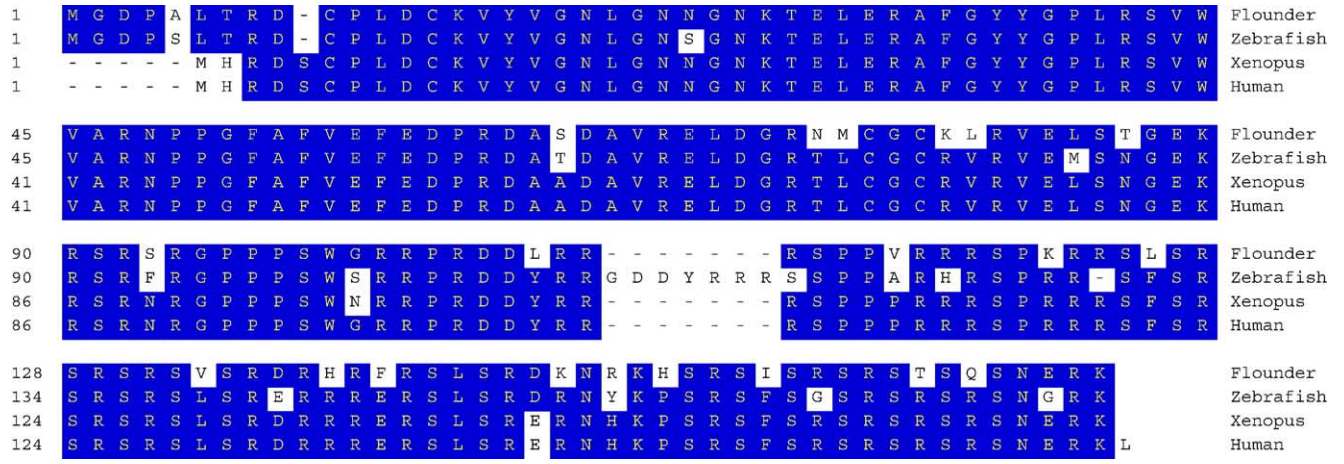

Fig. 2. Multiple sequence alignment of the deduced amino acid sequences of Srfs3 genes showing high levels of sequence identities. Sequences used for the alignment include SFRS3 of Japanese flounder, human (AAP36394), *Xenopus tropicalis* (AAH76942), and *Danio rerio* (AAH71322). The alignment was performed with the Clustal W program. The shaded areas are conserved residues.

20 min, 0.2× SSC two times at 45 °C for 40 min each, and finally in 50 mM PBS twice at room temperature for 20 min each. Nonspecific immunoreactivity in the tissues was blocked by incubating the larval specimens in 1% BSA and 0.4% Triton X-100 in PBS for 1 h at 37 °C. Larval specimens were then incubated for 4 h with anti-DIG-Fab-AP conjugate diluted 1:1000 (Boehringer Mannheim) at 37 °C. Finally, excess anti-DIG-Fab-AP was removed by five rinses (20 min) in PBS, and signals were visualized by incubation in Fast Red Detection Solution (Boehringer Mannheim) followed by a final two washes in PBS for 10 min each.

2.6. Histological processing and image collection

Whole mounts were observed and photographed using an Olympus microscope. Selected specimens were embedded in agarose gel and immersed in PBS containing 30% sucrose overnight. Then, the larval specimens were cut into serial frozen sections with a thickness of 8 μm for histological observations. Images were collected by using a CDC camera with software Motic Images Advanced 3.0 (Micro-optic Industrial Group, China). Images were later compiled and labeled using Motic Images Assembly 1.0 (Micro-optic Industrial Group. China) and Adobe Photoshop 6.0 (Adobe Systems Inc., USA).

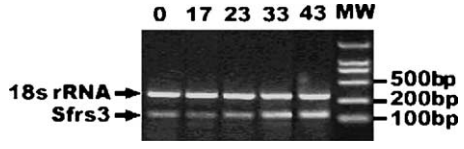

Fig. 3. Analysis of SFRS3 gene expression using RT-PCR. The time points of samples are indicated at the top of the gel: 0, embryo; 17, 23, 33, and 43 are 17, 23, 33, and 43 days after hatching, respectively. The positions of the RT-PCR products of SFRS3 and 18S rRNA are indicated on the left margin of the gel, and the molecular weight (MW) are indicated on the right margin of the gel.

3. Results

Suppression subtraction hybridization was conducted to identify upregulated transcripts during metamorphosis. RNA from Pre-metamorphosis stage (17 dph larvae) was subtracted from Stage E during metamorphosis (23 dph larvae). A total of 50 transformants with inserts were initially sequenced. One of the clones containing a 261-bp insert was identified to be SFRS3, the splicing factor, arginine-serine rich 3, by BLAST similarity comparison after initial sequencing. Further efforts focused on structural characterization and expression analysis of this gene.

The entire cDNA of the SFRS3 was obtained by PCR screening of a cDNA library made from RNA of Japanese flounder isolated from head tissues at Stage E of development. Its entire sequence was deposited in the GenBank with accession number ofAY197342. Sequencing analysis revealed that the flounder SFRS3 included an open reading frame of 504 bp encoding 168 amino acids. The cDNA had

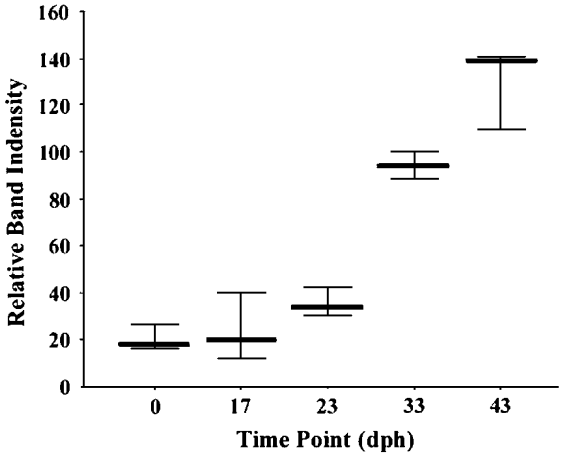

Fig. 4. Quantitation of RT-PCR analysis for SFRS3 expression. RT-PCR reactions were repeated for three times and the data are presented as relative band intensity of the SFRS3 over that of 18S rRNA.

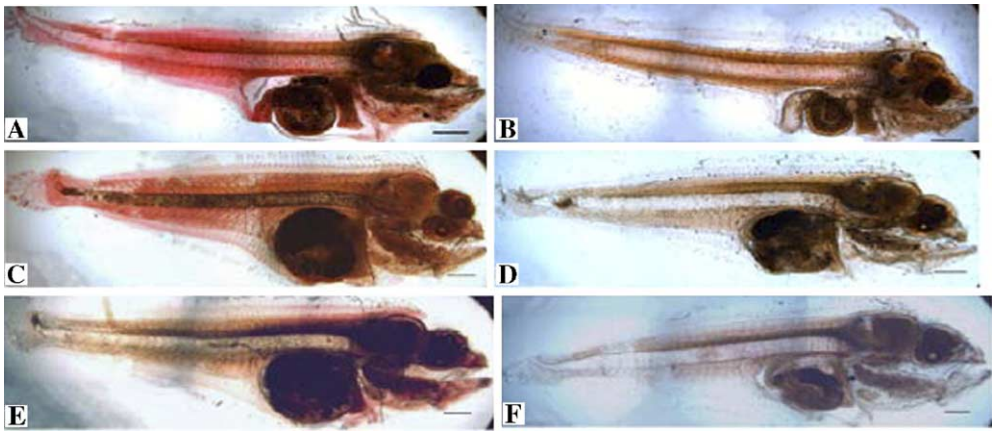

Fig. 5. Whole mount in situ hybridization. Japanese flounder from various stages as specified were treated for whole mount in situ hybridization as detailed in Materials and methods. Hybridization signals were reflected as red color. A, stage E with antisense probes; B, stage E with sense probe (negative control); C, stage G with antisense probes; D, stage G with sense probe; E, stage H with antisense probes; and F, stage H with sense probes. Scale bar: 500 μm.

a 138-bp 5′-untranslated region (URT), and a 622-bp 3′-UTR. A typical polyadenylation signal sequence AATAAA existed 13 bp upstream of the poly A tail (Fig. 1).

The SFRS3 gene is highly conserved through evolution with high contents of arginine and serine. The 168 amino acid protein contains 35 arginine and 23 serine residues. The protein is highly basic because of the high content of arginine with a PI of 11.5. As shown in Fig. 2, the flounder SFRS3 shares high levels of amino acid identities to those from other species, with over 82% amino acid identity to the human SFRS3, demonstrating great level of evolutionary conservation. Although the function of the flounder SFRS3 awaits to be demonstrated, its high similarity with the mammalian SFRS3 including the conservation of functional motifs, such as the eukaryotic RNA recognition motif (RRM) at positions 14–87 and an arginine/serine-rich region at positions 90–168 strongly suggest conservation of its functions as well.

The identification of SFRS3 through suppression subtraction hybridization suggest its upregulation during metamorphosis. In order to confirm its upregulated expression, a semi-quantitative RT-PCR was conducted to assess the levels of its expression during the course of metamorphosis. As shown in Figs. 3 and 4, the level of SFRS3 mRNA in the flounder head increased sharply during metamorphosis. Its expression was higher at the Stage E than at the pre-metamorphosis stage. Higher levels of SFRS3 expression were observed at 23 days after hatching, and its expression was much higher at 33 and 43 days after hatching (Figs. 3 and 4); as compared to the expression in the embryo, the level of SFRS3 gene expression increased four times at stage G, and seven times at stage H.

In order to assess spatial expression of SFRS3, whole mount in situ hybridization was performed using samples collected at various stages of development. As shown in Fig. 5, at stage E, SFRS3 gene was expressed all over the whole body from the head to caudal fin although expression in the head was relatively weak. As metamorphosis progressed, the expression of SFRS3 appeared to increase in the anterior regions, but decrease in the posterior regions. At stage G, SFRS3 was expressed uniformly across the whole body. However, as the metamorphosis reached stage H at 43 days after hatching, the SFRS3 was mainly expressed in the head region, whereas expression in the posterior portions of the body was very low (Fig. 5). This pattern of hybridization using whole mount samples was consistent with the result of

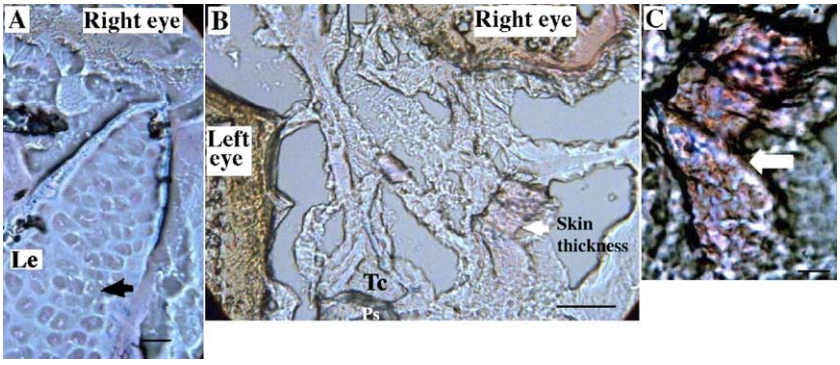

Fig. 6. Transverse sections through eye at Stage G. A, showing hybridization within lateral ethmoid; B, showing hybridization in the “skin thickness”; and C, enlarged view of stronger hybridization signals within the “skin thickness” area as shown in B. *Le* lateral ethmoid, *Tc* trabecular, *Ps* parasphenoid. Scale bars: 10 μm (A), 50 μm (B), 10 μm (C).

RT-PCR that indicated enhanced expression in the head as metamorphosis progressed to stage H.

Using in situ hybridization of sectioned tissues, we attempted to analyze expression of SFRS3 surrounding the areas where "skin thickness" tissues expand rapidly on the right side below the eye that was believed to push the movement of the right eye toward left. At the stage G, SFRS3 expression was detected in the lateral ethmoid (Fig. 6A), but greater levels of expression was detected within the "skin thickness" tissues (Figs. 6B, C) than in surrounding areas. It is likely that expression of SFRS3 was correlated with rapid cell division within the area.

4. Discussion

All SR proteins contain at least one RNA recognition motif (RRM) at the N-terminus and a region of varying length rich in alternating serine and arginine residues at the C-terminus (RS domain) [28]. In the present study, a full-length cDNA encoding SFRS3 of Japanese flounder was isolated. The putative flounder SFRS3 protein contained an arginine/serine-rich region and a Eukaryotic RNA Recognition Motif (RRM), and is structurally highly conserved with its orthologues in other organisms. All its structural features suggested its conserved functions.

The cDNA was initially identified from suppression subtraction hybridization during metamorphosis, indicating its upregulation during development involving metamorphosis processes. RT-PCR analysis as well as in situ hybridizations revealed its upregulation in the head starting at pre-metamorphosis stage and reached high levels at stage H. Relatively little information is available from the literature concerning the role of SFRS3 gene during development. The sharp increase of SFRS3 expression in the head, particularly in the areas where rapid tissue expansion might be happening during metamorphosis [29–32], could suggest potential roles of SFRS3 during metamorphosis of flounder. However, it is more likely that high levels of SFRS3 expression could be correlated with rapid cell proliferation. SFRS3's expression was reported to be regulated in late G1 or early S phase during cell cycle, and the promoter of SFRS3 gene contains two consensus binding sites for E2F [33]. Further studies are required to understand the functions of upregulated expression of SFRS3 gene in relation to metamorphosis of flounder.

Acknowledgements

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