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ABUNDANT EXPRESSION OF NUCLEOSOME ASSEMBLY PROTEIN 1 (NAP1) GENE IN GOLDFISH SCALE WITH LATERAL LINE

LiHua LI[1], Hironobu KATSUYAMA[2], Son Ngoc DO[1], Masayuki SAITO[1], Hideji TANII[1] and Kiyofumi SAIJOH[1]

[1]*Department of Environmental and Molecular Bio-Informatics, Kanazawa University School of Medicine, 13-1 Takaramachi, Kanazawa 920-8564, Japan*

[2]*Department of Public Health, Kawasaki Medical University, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan*

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ABSTRACT — To explore the possible utilization of goldfish scale in monitoring environmental toxicants as well as that for calcified tissue research, characteristics of cells in the scale were examined by identifying genes differentially expressed in those cells. Subtraction cloning was subjected to RNAs between scale − gill (SG), gill − scale (GS) and scale with lateral line − scale without lateral line (LS). Total numbers of 4, 6, and 9 clones were isolated respectively from SG, GS and LS pools. Blast search of their sequence showed only low homology to other fish sequences so that 5' rapid amplification of cDNA (5'RACE) was applied to the obtained 5' terminal side of each sequence. Among 19 clones, LS3 alone showed the high homology to zebrafish nucleosome assembly protein 1 (NAP1) and the rest of 18 was not yet identified. In situ hybridization confirmed that faint expression of NAP1 mRNA was observed in cells along the ridge of the scale without lateral line. On the other hand, in small cells not along the ridge of the scale with lateral line, very intense hybridization was found as expected. They were abundant at the surrounding area of the lateral line tube. NAP1 plays an enhancing role on gene expression by promoting nucleosome assembly. Thus, cell viability in the scale with lateral line seemed to be higher than that in the scale without lateral line. The scale can be a good model to monitor the effects of environmental pollutants because simultaneous observation against cells with different cell viability is available through comparison between cells in the scale with or without lateral line.

KEY WORDS: Goldfish, Nucleosome assembly protein 1, scale, lateral line, In situ hybridization

INTRODUCTION

Teleost scales are recently utilized for research exploring not only vertebrate ectodermal/dermal evolution (Sharpe, 2001; Yelick and Schilling, 2002) but also developmental and regenerating mechanisms of calcified tissues (Nishimoto *et al*., 1992; Lehane *et al*., 1999; Suzuki and Hattori, 2002; Wagner *et al*., 2003). For the latter, it is important that, in order to analyze the molecular mechanism of microgravity-induced bone loss, small bony fish medaka *Oryzias latipes* experienced long-term space flight (Wagner *et al.*, 2003). The existence of scales in fish is related to the protective functions of the vertebrate integument, and because of increasing swimming performance, each scale is small and thin (Yamada, 1961; Wagner *et al*., 2003). However, the scale can contain about 20% of the total body calcium in some fish as in the case of goldfish. They appear to represent an internal calcium reservoir and their mineral is supposed to be more easily available than that of the bone (Wagner *et al*., 2003). Each scale is inserted in its own pocket between the superficial dermis and the epidermis so that it can be lost either spontaneously or by external stimuli. Even when it is not lost, its surface is easily wounded (Yamada, 1961; Wagner *et al*., 2003). Thus, formation/resorption of the

Correspondence: Kiyofumi SAIJOH (E-mail: saijohk@med.kanazawa-u.ac.jp)

scale have been focused on in relation to either external physical (Sire *et al*., 1990; Bereiter-Hahn and Zylberberg, 1993; Wagner *et al*., 2003), or chemical stimuli (Nishimoto *et al*., 1992; Lehane *et al*., 1999; Suzuki *et al*., 2000; Suzuki and Hattori, 2002; Wagner *et al*., 2003). It is conceivable that the scale is suitable to monitor effects of environmental pollutants. However, the characteristics of each cell located in the scale are still unclear, excluding multi-nuclei cells that appear in the wounded region (Sire *et al*., 1990).

The scales with lateral line include a sensory nerve terminal. Similar to acoustic nerve, this terminal is a ciliar cell dipped in endolymph. However, it senses water flow and vibration (Kuiper, 1956) but not sound (Dijkgraaf and Kalmijin, 1963). Thus, lateral line nerve is similar to the nerve for deep sensation in mammals. To sense water flow and vibration, the lateral line tube wall where the terminal locates is maintained very thin. That is, there should be very strict mechanisms for the maintenance of the shape of the tube within the scale. Thus, from the point of view of sensory nerve development, the scale can be a very attractive model.

In order to clarify whether the scale is a useful tool for the assessment of effects of environmental pollutants and/or for that of neuronal development, we tried to identify the genes differentially expressed in the scale.

MATERIALS AND METHODS

Goldfish scale specimen

Mature goldfish, *Carassius aurantus*, were purchased from a local commercial source. Their scale (approximately 5 mg each) (Fig. 2A and B) and gills were obtained under anesthesia with ethyl 3-aminobenzoate methanesulfonic acid (Aldrich, Milwaukee WI).

Subtraction cloning

Total RNA was purified from the scale and gill using acid/guanidium/phenol/chroloform methods (Davis *et al*., 1994). After polyA(+) selection, the specimen was subjected to subtraction cloning using PCR-Select cDNA Subtraction kit (Clontech. Palo Alto, CA). The subtraction was made in scale – gill (SG), gill – scale (GS) and scale with lateral line – scale without lateral line (LS). Since all the cells in the scale without lateral line existed in the scale with lateral line, subtraction SL was not performed. The products were digested with EagI (Toyobo, Osaka, Japan) and cloned into NotI (Toyobo) digested pBluescript (Stratagen, La Jolla, CA). The sequence of inserts was confirmed using BigDye Terminater v3.0 cycle sequencing kit (Applied Biosystem, Foster City, CA). The obtained sequence was subjected to BLAST homology search.

5' rapid amplification of cDNA (5'RACE)

Isolated clones were not always homologous to fish sequences registered to Genebank, so that 5'RACE was applied in order to obtain the coding sequence using BD SMART™ RACE cDNA amplification kit (BD Bioscience Clontech). After cloning into pGEM-T vector (Promega, Madison, WI), the inserts' sequence was confirmed as described above.

In situ hybridization

The scales removed were immediately immersed in 1% glutalaldehyde. After rinsing in distilled water, a whole scale was treated with 10 μg/ml proteinase K (sigma, St Louis, MO) at 37°C for 30 min, treated with 0.2 M HCl, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamien (pH 8.0), dehydrated with ethanol series, and air dried. After prehybridization with hybridization a solution containing 50% formamide, 10% dextran sulfate, 1 × Denhardt's solution (Davis *et al*., 1994), 600 mM NaCl, 0.25% sodium dodecyl sulfate, 150 μg/ml yeast tRNA at 50°C for 2 hr, each scale was allowed to hybridize to approximately 0.5 μg/ml of the cRNA probe at 50°C overnight. The bi-directional cRNA probes used were synthesized for nucleosome assembly protein 1 (NAP1) using either SP6 or T7 RNA polymerase (Takara, Tokyo, Japan) and the DIG labeling kit (Boehringer, Mannheim, Germany). Sense cRNA served as a negative control. The scale was washed briefly in 5 × sodium citrate/chrolide buffer (SSC) at ambient temperature, in 50% formamide and 2 × SSC at 50°C for 30 min, and treated with 10 μg/ml RNaseA at 37°C for 30 min. Hybridized probes were detected with the Nucleic Acid Detection kit (Boehringer). The scale was also subjected to HE staining.

RESULTS

Totals of 4, 6, and 9 clones were isolated respectively from the SG, GS and LS pools. Blast search of their sequence homology showed that none of the sequence was matched to mRNA sequences. However, all of the sequences showed homology with very low identities to the fish sequences in linkage groups (Table 1). The sequences of zebrafish, *Danio Rerio*, Japanese bitterling, *Rhodeus suigensis*, and carp are common in the database, whereas that of goldfish is not. The sub-

traction kit utilized oligodT as a primer to synthesize the first strand cDNA, hence obtained cDNAs were supposed to mainly include 3' terminal side but not 5' terminal side, ie., it was less possible that they included the coding sequences. Such a situation seemed to be the main reason why their sequence did not always show high homology even against the fish mRNA sequences. Thus, we utilized 5'RACE to obtain the flanking 5' sequence. Even after 5'RACE cloning, the obtained sequences did not always show high homology to the registered sequences. LS3 alone showed high homology to zebrafish nucleosome assembly protein 1 (NAP1), being 89% in mRNA (Fig. 1A) and 95% in putative protein (Fig. 1B). In order to confirm differential expression and to identify which cell was responsible for this abundant expression, in situ hybridization was applied.

There were several types of the cells in the scale (Fig. 2A and B), whereas their identity was not always established and they are called scleroblasts (Yamada, 1961) in general (Fig. 2A). NAP1 expression was faintly positive in the cells along the ridges in the scale without lateral line (Fig. 3E). Only a small number of cells not along the ridge showed faint positivity. On the other hand, the small cells not along the ridge were intensely stained in the scale with lateral line (Fig. 3C). Moreover, these cells were abundant at the area surrounding the lateral line tube (Fig. 3A).

DISCUSSION

Teleost scale is currently utilized for research on developmental and regenerating mechanisms of calcified tissues (Nishimoto *et al*., 1992; Lehane *et al*., 1999; Suzuki and Hattori, 2002; Wagner *et al*., 2003). Osteoporosis is one of the important social burdens of such requirement. In order to avoid complications like fracture, bed-ridden, etc., biochemical markers like alkaliphosphatase (ALP) and Gla-osteocalcin for formation and type I collagen cross-linked N-telopeptide and pyridinoline for resorption are utilized to detect rapid bone loss at an early stage before significant bone

Table 1. Isolated cDNAs and their homology to the sequences registered to the database.

Clone	insert length (base)	the sequence with homolgy	Identities (base/base)	*Accession Number
GS1	155	Zebrafish CH211-244H4	144/155	AB331177
GS2	400	Zebrafish DKEY-7C18	100/116	AB331178
GS3	390	Carp carp-C114	83/88	AB331179
GS4	211	Zebrafish DKEY-23L19	77/88	AB331180
GS5	155	Zebrafish CH211-220I18	23/23	AB331181
GS6	390	Zebrafish CH211-220I18	23/23	AB331182
GS7	389	Zebrafish CH211-172B12	20/20	AB331183
SG1	367	Carp carpf-C114	83/88	AB331173
SG2	289	Zebrafish DKEY-48F17	29/31	AB331174
SG3	459	Zebrafish CH211-197E11	22/22	AB331175
SG4	289	Zebrafish CH211-273K1	19/19	AB331176
LS1	458	Danio rerio zgc:64222	104/110	AB331184
LS2	460	Zebrafish DKEY-10P5	74/86	AB331185
LS3	209	Zebrafish CH211-237E12	73/82	AB331186
LS4	159	Zebrafish DKEY-117M4	72/83	AB331187
LS5	390	Zebrafish DKEY-23L19	71/72	AB331188
LS6	114	Zebrafish CH211-276C7	44/47	AB331189
LS7	460	Zebrafish DKEY-230N6	31/32	AB331190
LS8	389	Zebrafish CH211-135B6	26/26	AB331191
LS9	114	Zebrafish CH211-276C7	22/24	AB331192

* accession No for DNA Data bank of Japan (http://www.ddbj.nig.ac.jp). 5'RACE clone of LS3 encoding NAP-1 like protein 1 is available as AE329667.

loss occurs (Ross, 1999). However, significant associations between markers and bone loss rates are not always confirmed (Ross, 1999). On the other hand, the receptor activator of the nuclear factor kappaB (RANK) and RANK ligand (RANKL) interaction (Schett *et al*., 2004), and its modification by a decoy receptor osteoprotegerin (OPG), PTH, interleukins, etc (Huang *et al*., 2004; Schett *et al*., 2004) is found to be important using cultured cells like osteoblastic MC3T3-E1, bone marrow stroma cells with or without stimulation, osteogenic sarcoma cells and giant-cell tumor-derived osteoclast, etc (Ross 1999; Hu *et al*., 2004; Huang *et al*., 2004; Schett *et al*., 2004; Varga *et al*., 2004). However, these cells are not always suitable for investigating osteoblast/osteocyte/osteoclast interaction. It is absolutely necessary to examine the precise relationship between these cells and bio-active molecules in order to clarify the underlying mechanism of bone formation/resorption and to utilize these markers. However, encasement of these cells in calcified tissue makes research difficult. Thus, it is attractive if the scale, where morphological observation is easy because of its transparency and softness, is available to explore the mechanism of bone formation/resorption.

Its utilization to estimate effects of environmental pollutants is also attractive. In addition to experimental teleosts, wild teleosts are supposed to be a good index to assess environmental pollution. We already utilized zebrafish to clarify the effect of environmental estrogen and found that environmental estrogen caused craniofacial developmental anomaly (unpublished data). Its gene sequence is well-established so that zebrafish is suitable to explore alteration in gene/protein expression by environmental stimuli but too small to obtain sufficient amount of RNA. Carp is too big to manipulate. Moreover, both of them are very expen-

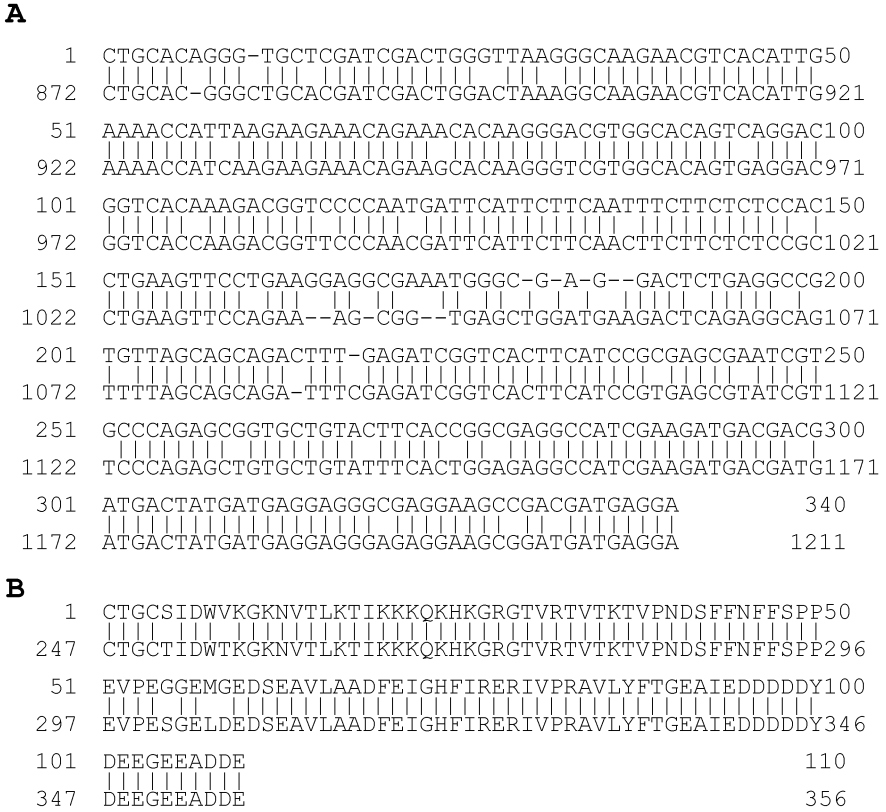

Fig. 1. Homology of LS3 sequence against zebrafish NAP1. Nucleotides (A) and amino acids (B) with vertical bars are identical. This sequence was utilized to develop probes for in situ hybridization.

sive. Goldfish is cheap and its size is suitable to manipulate. Thus, we are trying to utilize goldfish scale to monitoring the effect of environmental pollutants even when its genetic background is not always clarified.

Although a total of 19 cDNA clones were isolated, only one clone from the LS pool corresponded to cDNA with known function. For the rest of the 18 clones, it is necessary to obtain full-length cDNA to identify what kinds of protein were encoded. It is at least possible to say that gene expression in the scale was in a tissue-specific manner. Moreover, abundant expression of NAP1 in the scale with lateral line already proved the utility of the scale in toxicological research as well as in calcified tissue research. NAP1 promotes nucleosome assembly, inhibits the accessibility of histone H3 and H4, and enhances coactivator-mediated gene expression (Eckey *et al.*, 2007). Thus, it is suggested that cell viability in the scale with lateral line was higher than that in the scale without lateral line, which was probably necessary for the maintenance of lateral line tube nerves. The scale can be a good model to monitor the effects of environmental pollutants because simultaneous observation of cells with different cell viability is available through comparison between cells in the scale with or without lateral line. It is also known that NAP1 is downstream to vitamin D receptor (Eckey *et al.*, 2007). Thus, the cells in the scale might share a similar character with osteocytes/osteoblasts, which allow them to be a good model in the research of calcified tissue turnover.

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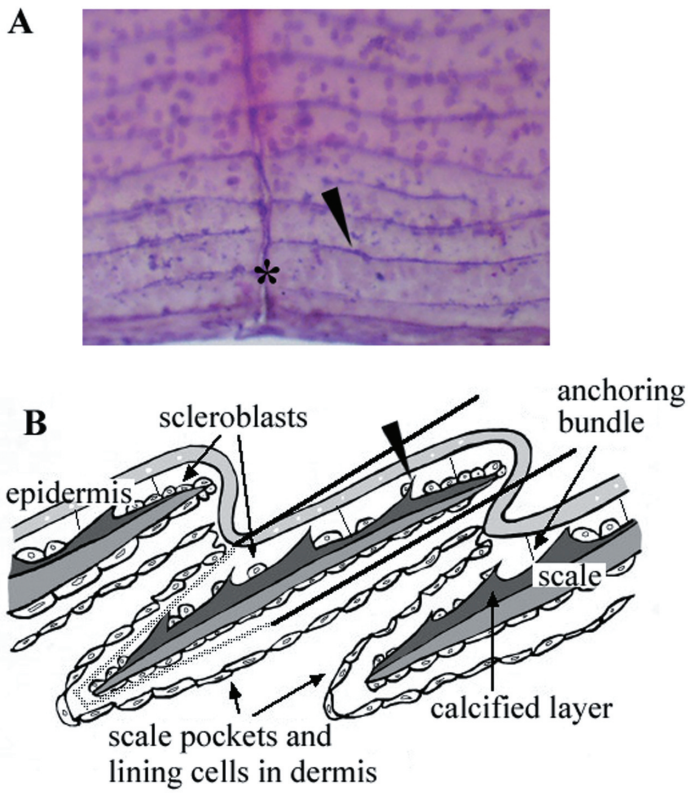

Fig. 2. HE staining of the scale (A) and its schematic diagram (B). Magnification: ×200 (A). Each scale between solid bars was removed (B). Asterisk, grooves considered to serve flexibility to the scale; and solid arrow head, ridges.

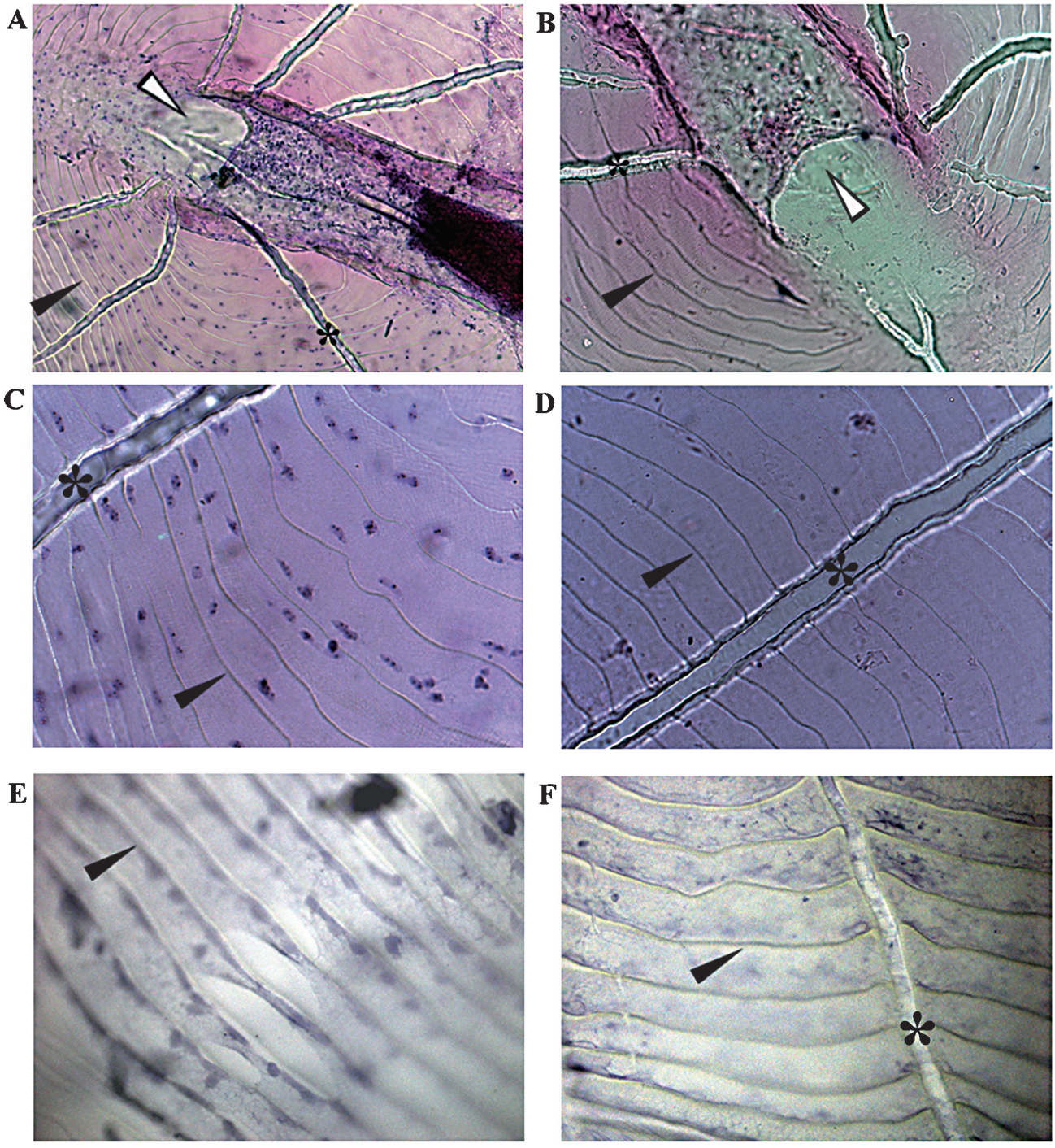

Fig. 3. In situ hybridization for NAP1.
Probed with anti-sense NAP1 (A and C, the scale with lateral line; and E, the scale without lateral line) and sense NAP1 (B and D, the scale with lateral line, and F, the scale without lateral line) cRNAs. Sense cRNA served as a negative control. Asterisk, grooves; solid arrow head, ridges; and open arrow head, lateral line tube. Magnification: ×100 (A and B) and ×200 (C, D, E, and F).

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