Molecular cloning and expression of *Ad4BP/SF-1* in the serial sex changing gobiid fish,
Trimma okinawae

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Abstract

Involvement of *Ad4BP/SF-1* was investigated using the serial sex changing goby *Trimma okinawae*. First, a cDNA encoding *Ad4BP/SF-1* was cloned from ovarian follicles. The open reading frame of goby *Ad4BP/SF-1* encodes a protein of 489 amino acids. *Ad4BP/SF-1* was expressed in gonadal tissues, brain and kidney. Secondly, transcript levels of *Ad4BP/SF-1* were measured in the ovary and testis of the same individuals. Expressions were related to sexual phases. Moreover, ovarian expression of *Ad4BP/SF-1* increased during vitellogenesis and declined sharply during the post-vitellogenic period. Finally, the expression profile of *Ad4BP/SF-1* was measured during serial sex change in the ovary. *Ad4BP/SF-1* increased in parallel with the onset of the female-phase and decreased as females became male. Therefore, *Ad4BP/SF-1* probably acts as an important indirect regulator of oocyte growth and maturation at female-phase of serial sex changing gobiid fish *Trimma okinawae*.

Keyword: Ad4BP/SF-1; sex-changing fish; gonadal development and maturation; gobiid fish.

Introduction

Trimma okinawae is a small orange-colored goby distributed from Kagoshima south to the Ryukyu Islands, Japan. Gonads of mature fish can simultaneously contain ovarian and testicular tissues [1]. Field observations revealed that a dominant male controls a harem of several females. Removal of this dominant male results in sex change of the largest female, which become the new dominant male in the harem. If subsequently placed with a larger male, the once dominant male reverts back to the female [2]. Thus, since serial sex change can be socially manipulated, the goby provides an excellent animal model to elucidate the endocrine mechanisms controlling sex change.

Steroid hormones produced by the gonads play a crucial role in ovarian function, sex differentiation and sexual maturation and behavior in vertebrates in general [3-6]. In hermaphrodite fishes, steroid hormones also play an important role in natural and experimentally induced sex change [7-9]. Our specific concern is in the physiological function of steroid hormone and the regulation of its synthesis during sex change. Steroid hormones are synthesized in the gonad and brain by many enzymatic steps using cholesterol as a substrate. Recently, we confirmed the presence of three steroid organic enzymes (P450 cholesterol-side-chain-cleavage (P450scc), 3β -hydroxysteroid dehydrogenase (3β -HSD) and P450 aromatase (P450arom)) in gonads of *Trimma okinawae* during serial-sex change by immunohistochemical method [10, 11]. These results suggest that steroidogenic enzymes should be important for development of gonad and serial sex change. However, it remains unclear how sex change affects gonadal endocrine mechanisms. To obtain detailed information about gonadal steroidogenic hormone synthesis during sex change, analysis of the transcriptional

regulators of steroidogenic enzymes is required.

Ad4 binding protein (Ad4BP)/steroidogenic factor-1 (SF-1), an orphan nuclear receptor protein, was identified as a steroidogenic tissue specific factor in mammals [12-15]. This protein also has high homology to *Drosophila fushi tarazu-factor 1* (*FTZ-F1*); hence, in some of the species it was also named as *FTZ-F1* [13, 16-20]. The primary structure of the gene has been characterized in many species including teleosts [13, 21-23]. In mammals, Ad4BP /SF-1 functions as a transcription factor involved in the regulation of steroidogenic enzymes including P450_{scc}, 3 β -HSD and P450_{arom} [24-28]. Recently, studies in teleost demonstrated that Ad4BP/SF-1 regulates *P450arom* expression by binding to its promoter region [16, 22]. Consequently, we focused on Ad4BP/SF-1 expression during sex change in *T. okinawae*.

In this study, we first isolated *Ad4BP/SF-1* cDNA of the serial sex changing gobiid fish, *Trimma okinawae*, using 5'- and 3'-RACE. Subsequently, transcript levels of *Ad4BP/SF-1* were measured in gonadal tissues of male- and female-phase fish using quantitative real-time RT-PCR. Finally, changes in *Ad4BP/SF-1* expression were examined during the natural ovarian cycle and sexual transitions (from male to female and female to male).

Materials and methods

Experimental animals

Animals were collected from their natural habitat in Makurazaki, Kagoshima prefecture, Japan, and then kept in the laboratory. Fishes were maintained under constant conditions (seawater at 27°C, 14 hour Light: 10 hour Dark) in indoor tanks.

Fish were fed commercial pellet and brine shrimp. Sex was determined by genital papillae examination [2, 29]. Samples for different sexual phases and ovarian cycle study were collected after spawning to prevent contamination by sex changing individuals [30]. We use the male- and female-phase fish 3days after spawning to make samples of various stages of sex change (male to female (M→F) and female to male (F→M)) according to established methods [2].

Sample collection and RNA extraction

Adult fish were sedated in an ice bath and sacrificed by decapitation. Testis and ovary were removed from same individuals described previously [1]. Total RNA from gonads was isolated using the RNeasy mini kit with the RNase-free DNase set (Qiagen) by following supplier's protocols. Poly (A)⁺ RNA was extracted from frozen ovary with the FAST Track 2.0 kit (Invitrogen, SanDiego, CA, USA).

Isolation of *Ad4BP/SF-1* cDNA

The goby *Ad4BP/SF-1* cDNA was cloned and sequenced by RT-PCR and rapid amplification of cDNA ends (RACE) analysis.

Two highly conserved regions in the DNA-binding domain and the Ad4BP/SF-1 box of *Ad4BP/SF-1* were selected as degenerate primers for RT-PCR: sense-5'TGTCC (A/G) GTGCGG (A/T) GACAA-3'; antisense-5'GCCC (T/G) GTCTC (T/G) GTCTC (T/G) CTTGTACAT-3'. As a template for RT-PCR, first strand cDNA was synthesized from 1 µg of ovarian total RNA using SuperScript II RNase H⁻ reverse transcriptase (Gibco BRL) with an oligo- (dT)₁₈ primer. RT-PCR conditions were 94°C for 1 min, 52°C for 1 min, 72°C for 1.5 min for 30 cycles. The resultant PCR amplicon was

gel-purified and cloned into pGEM-T Easy vector (Promega).

Based on the sequence information of the presumptive *Ad4BP/SF-1* amplicon, RACE procedures were performed to isolate the 5' and 3' ends of the cDNA. As a template for RACE, adapter ligated double strand cDNA was synthesized using the Marathon cDNA amplification kit (Clontech) according to manufacturer's protocol with the following modification; the anchor primer of oligo- (dT)₁₈ was changed to random hexamer primers.

5'-RACE was carried out using 5GSP1 (5'-CTGCACGCACCGCTTCCAGGCG-3') and adapter primer 1 (5'-CCATCCTAATACGACTCACTATAGGGC-3'; Marathon cDNA amplification kit, Clontech) under the following conditions for 25 cycles: 94°C 30 s, 68°C 4 min. Nested amplification was conducted using a 100-fold dilution of the first reaction, with 5GSP2 (5'-TGAGACATTTCTGGAACCGACA-3') and adapter primer 2 (5'-ACTCACTATAGGGCTCGAGCGGC-3'; Marathon cDNA amplification kit, Clontech). PCR conditions were 94°C for 30 s, 70°C for 4 min for 20 cycles. The resultant amplicon was purified and subcloned.

3'-RACE was carried out using 3GSP1 (5'-CTTCAAGAGGACAGTACAGAAC-3') and adapter primer 1 under the same condition as the 5'-RACE first amplification. The second nested amplification was conducted using 3GSP2 (5'-ACAAGACTCAGCGGAAGAGGTG-3') and adapter primer 2 under the touch-down PCR program (5 cycles: 94°C for 30 s, 72°C for 4 min; 5 cycles: 94°C for 30 s, 70°C for 4 min; 20 cycle 94°C for 30 s, 68°C for 4 min). The longest PCR product was purified and subcloned.

Finally, the complete open reading frame (ORF) of the goby *Ad4BP/SF-1* was

generated from ovarian RNA by RT-PCR (25 cycles, 94°C for 15 s, 68°C for 1 min) with primers targeting the untranslated portions immediately upstream (3GSP3) and down stream (5GSP3) of the ORF (Fig. 1), using high-fidelity DNA polymerase (KOD-plus- DNA polymerase; TOYOBO Co., LTD.). The resulting product was cloned into pBluescript II KS- (Stratagene, Land Jolla, CA) and sequenced in both directions.

DNA sequence analysis

Sequencing was performed in both directions with an Applied Biosystem model 377 sequencer after labeling with a dye terminator cycle sequencing kit (Applied Biosystems, Foster city, CA, USA) using vector and gene-specific primers. Sequence analysis was performed using DNASIS software (Hitachi Co, Ltd., Tokyo. Japan). Alignment of multiple protein sequence was performed using the ClustalW multiple sequence alignment program. Homology value (percent of amino acid sequence identity) was calculated by pair wise alignment.

Analysis of goby *Ad4BP/SF-1* tissue expression by RT-PCR

Single-stranded cDNA was synthesized from 0.25 µg of oligo(dT)-primed total RNA isolated from various tissues using Omniscript reverse transcriptase (Qiagen). PCR analysis of *Ad4BP/SF-1* (sense-5'-GCTGTCTCATAACTGCTGGTC-3'; antisense-5'-TCTCGATCAGCAGGTTGTTG-3') and *β-actin* (sense-5'-TTCTACAACGAGCTGCGTGT-3'; antisense-5'-TGTCAGGATCTTCATGAGGTA-3') was carried out under the following conditions: 30 cycles at 92°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Ten-microliter aliquots of the PCR reactions were electrophoresed on 1.5% agarose gels and the gels

were stained with ethidium bromide to visualize the band.

Real-time quantitative RT-PCR of goby *Ad4BP/SF-1*

Goby *Ad4BP/SF-1* transcript abundance was determined in the ovary during the spawning cycle and sex change by real-time quantitative RT-PCR (rtq-PCR) methods described previously [30, 31]. Briefly, 500 ng of total RNA was reverse transcribed in a 20 μ l reaction volume using random hexamer primers and Omniscript reverse transcriptase (Qiagen). The standard copy number of *Ad4BP/SF-1* transcripts was estimated based on molecular weight and absorbance of goby *Ad4BP/SF-1* plasmid. Two sets of primers were designed (rtq F1; 5'-AAGTTCATGCTCCTCTTCAAC-3', rtq R1; 5'-TCCACGAACGCCTGGTT-3') using Primer Express software (version 1.5, PE Applied Biosystems). Dilution templates of each triplicate were tested in 25 μ l of PCR mixture containing 2X SYBER Green master mix (PE Applied Biosystems) and 0.3 μ M (final concentration) of each primer. Then, one initial denaturation at 95°C for 15 min, 45 cycles of 94°C for 15 s, 52°C for 15s, and 72°C for 30 s were completed. A final extension at 60°C for 5 min was carried out for the dissociation curve analysis. The PCR amplifications and fluorescent detection were performed with the ABI Prism Sequence Detector 7000. The contamination of genomic DNA in the sample was checked by performing a control reaction having no reverse transcribed RNA.

Data analysis

Data are depicted as the mean $\pm$ SEM. The data were analyzed by student-*t* test or Kruskal-Wallis test (Statview, Abacus systems Inc.). A value of $P < 0.05$ was considered

as statistically significant.

Results

Cloning of goby *Ad4BP/SF-1* cDNA

The initial RT-PCR of goby ovary using the degenerate primers amplified a 275 bp fragment of cDNA. This amplified fragment was cloned and sequenced. A search in the GenBank database indicated highest sequence identity to other isolated vertebrate *Ad4BP/SF-1* sequences. Therefore, the gene-specific primers designed from this cDNA fragment were subsequently used for the RACE procedure. The 3' and 5' RACE PCR products included the open reading frame, but not the poly A⁺ tail (Fig. 1). The RT-PCR using ovarian RNA and primers 3GSP3 and 5GSP3 generated a single PCR product, and it contained the complete sequence. The nucleotide sequence of clones from this independent PCR confirmed those derived from the RACE clones.

The isolated cDNA encoded 489-amino acid protein. The deduced protein showed the greatest homology with *Ad4BP/SF-1* of other species, identity values being 92%, 91% and 59-69% with tilapia (GenBank accession no. **AB060814**), medaka (**AB016834**), zebrafish a (**AF014926**), goldfish (**AF526537**), catfish A (**AY014862**), *Xenopus* (**XLU05001**), chicken (**AB002404**), bovine (**D13569**) and mouse (**D90530**) (Fig.2). Figure 2 also shows that the DNA binding domain, the putative *Ad4BP/SF-1* box, the Region II and III and the activation factor-2 (AF-2) hexamer of goby *Ad4BP/SF-1* are highly conserved with other vertebrate homologues.

Tissue distribution pattern of goby *Ad4BP/SF-1*

The RT-PCR detected the presence of *Ad4BP/SF-1* goby transcripts in the gonadal tissues, brain and, at a very low level, in the kidney (Fig. 3). No transcripts were detected in the liver. In simultaneous reactions, *β-actin* was amplified with consistent intensity, indicating the uniformity of the template cDNA among the reactions.

Expression of *Ad4BP/SF-1* gene in ovarian and testicular tissues of male and female phases

The real time quantitative RT-PCR was performed to determine changes of *Ad4BP/SF-1* levels during the sexual phase as the one day before spawning (Fig. 4). In female-phase individuals, transcripts of *Ad4BP/SF-1* were higher in ovary than in testis. In contrast, few *Ad4BP/SF-1* transcripts were seen in the ovary of male phase. More transcripts were seen in the testis of male phase, but not in the testis of female phase.

Changes in transcription levels of *Ad4BP/SF-1* during the natural spawning cycle of *T. okinawae*

Changes in *Ad4BP/SF-1* expression at different stages of the ovarian cycle (spawning cycle) were examined using a quantitative real-time RT-PCR (Fig. 5). Expression levels of *Ad4BP/SF-1* were low soon after spawning. *Ad4BP/SF-1* transcripts increased from day 1, peaked at day 3 and returned to the original level on day 4. Elevated levels of *Ad4BP/SF-1* were correlated with the periods of active vitellogenesis.

Changes in transcription levels of *Ad4BP/SF-1* during serial sex change

Changes in transcription levels of *Ad4BP/SF-1* during sex changes from female to male and male to female were examined using a quantitative real-time RT-PCR (Fig. 6).

The *Ad4BP/SF-1* transcript level was high in ovary at day 1. The levels decreased significantly by day 2 of female to male sex change and remained low during the rest of the spawning cycle (Fig. 6a). In contrast, the *Ad4BP/SF-1* transcript level was low in ovary at day 1 during the male to female sex change. The expression levels steadily rose in ovaries during the male to female sex change from day 2 to day 10 (Fig. 6b).

Discussion

In this study, a cDNA encoding Ad4BP/SF-1 was isolated and characterized from ovary of the serial sex changing goby, *T. okinawae*. The cDNA possesses the typical features of the orphan nuclear receptor superfamily. Conserved regions that are essential for DNA- and ligand-binding domains showed high homology with other vertebrate *Ad4BP/SF-1* cDNAs [32]. In addition, activation function-2 (AF-2) hexamer showed 100% identity, indicating that goby clone can be classified as a member of Ad4BP/SF-1 family [33, 34].

The present study using RT-PCR analysis revealed that *Ad4BP/SF-1* transcripts were present in the ovary, testis, brain, and kidney. In contrast, no *Ad4BP/SF-1* expression was seen in the liver. This tissue distribution pattern including the absence of expression in liver agrees with that of earlier studies on medaka [16] and tilapia [22] and tends to indicate that goby *Ad4BP/SF-1* can be grouped with *Ad4BP/SF-1* rather than with *LRH-1/FTF* [14, 35].

Ad4BP/SF-1 transcripts mRNA expression was mainly observed in the active gonad of the corresponding sexual phase. Ovaries, but not testis, of female phase contained high levels of *Ad4BP/SF-1* transcripts. Similarly, Ad4BP/SF-1 transcripts were higher

in testis of the male phase than in female phase. Thus, *Ad4BP/SF-1* was normally required for the steroidogenesis and several P450 steroid hydroxylases in the active gonad [36, 37].

It has been shown that *Ad4BP/SF-1* is a transcriptional regulator for *P450arom* expression and activity in mammals [38] and fishes [16, 22]. Studies using medaka had already established that mutated oligomeric sequences of Ad4 could not bind specifically either to ovarian follicular nuclear protein or to *in vitro*-translated Ad4BP/SF-1 [16]. In tilapia, it was shown that oligonucleotide probes containing putative orphan nuclear receptor binding motifs (derived from promoter region of the aromatase gene) formed complexes with *in vitro*-translated Ad4BP/SF-1 and nuclear extracts of tilapia ovarian follicles [21]. High levels of *Ad4BP/SF-1* transcripts are correlated with active vitellogenesis similar to medaka [16] and tilapia [21]. Thus, in *T. okinawae*, *Ad4BP/SF-1* likely acts as a transcriptional regulator for *P450arom* during oocyte development (vitellogenesis).

Both *Ad4BP/SF-1* and *P450arom* transcript levels showed a sharp drop in the female phase ovary prior to spawning [30]. In teleosts, there is a distinct shift in ovarian steroidogenesis from estrogens to progestogens prior to final oocyte maturation. This steroidogenic shift is crucial for the oocytes to undergo final oocyte maturation. In most fishes, estradiol-17 β promotes vitellogenesis, while 17 α ,20 β -dihydroxy-4-pregnen-3-one (17 α ,20 β -DP) induces oocyte maturation [3]. In this shift, two steroidogenic enzymes, P450arom and 20 β -hydroxysteroid dehydrogenase (20 β -HSD), both of which are located in the granulosa cell layer of ovarian follicles, are important. Thus, there is a shift in steroidogenic enzyme activity from P450arom to 20 β -HSD, leading to rapid decrease in estradiol-17 β biosynthesis by

the post-vitellogenic ovarian follicle. One of the mechanisms responsible for this decreased activity of P450arom is the decrease in the level of *P450arom* transcripts. This occurs in the granulosa cell layer immediately prior to oocyte maturation. Recent *in vitro* incubation studies showed that both *P450arom* and *Ad4BP/SF-1* transcripts sharply declined and became undetectable when post-vitellogenic full-grown immature follicles were incubated with human chorionic gonadotropin (HCG) [21]. Taken together, these results suggest a role for Ad4BP/SF-1 probably in the transcriptional regulation of *P450arom* in fish ovarian follicles and perhaps gonadotropins assign such a major function. Further studies are required to determine whether Ad4BP/SF-1 of *T. okinawae* can specifically bind to its DNA motif (Ad4). It is also important to determine whether gonadotropins, in particular luteinizing hormone (LH), are responsible for the rapid decrease in both *P450arom* and *Ad4BP/SF-1* transcripts in the sex changing goby. Future studies should also identify the maturation-inducing hormone of this species.

In *T. okinawae*, *Ad4BP/SF-1* transcript levels in the ovary also varied with sex change. Significant increases in Ad4BP/SF-1 were observed in ovaries of individuals changing sex from the male to female phase. In contrast, *Ad4BP/SF-1* transcripts declined sharply in ovaries of fish changing sex from the female to male phase. These findings are of particular interest, since it was reported that in the protogynous wrasse, *Thalassoma duperrey*, rapid decline of serum estradiol-17 β levels is an initial trigger of sex change [9]. Our findings indicate a direct relationship between *Ad4BP/SF-1* expression and the female phase. In mammals, *Ad4BP/SF-1* gene knock out resulted in a lack of reproductive organs, suggesting that Ad4BP/SF-1 functions as a transcriptional factor involved in the regulation of steroidogenic enzymes as well as differentiation of steroidogenic tissues. Ad4BP/SF-1 is essential for ovarian differentiation in mammals

[24]. Consequently, *Ad4BP/SF-1* may play a similar role in normal ovarian function and development during sex change of *T. okinawae*. However, we did not observe Ad4BP/SF-1 expression in the testis during sex change. Future study is need to clarify the functional of Ad4BP/SF-1 in the testis.

In summary, these results suggest a role for *Ad4BP/SF-1* may act as an important indirect regulator of oocyte growth and maturation.

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Reference

- [1] Y. Kobayashi, T. Sunobe, T. Kobayashi, Y. Nagahama, M. Nakamura, Gonadal structure of the serial-sex changing gobiid fish *Trimma okinawae*, *Develop. Growth Differ.* 47 (2005) 7-13
- [2] T. Sunobe, A. Nakazono, Sex change in both directions by alternation of social dominance in *Trimma okinawae*, *Ethology* 94 (1993) 339-345.
- [3] Y. Nagahama, Endocrine regulation of gametogenesis in fish, *Int. J. Dev. Biol.* 38 (1994) 217-229.
- [4] Y. Nagahama, A. Matsuhisa, T. Iwamatu, N. Sakai, S. Hukada, A mechanism for the action of pregnant mare serum gonadotropin on aromatase activity in the ovarian follicle of the medaka, *Oryzias latipes*, *J. Exp. Zool.* 259 (1991) 53-58.
- [5] J. S. Richards, Hormonal control of gene expression in the ovary, *Endocr. Rev.* 15 (1994) 725-751.
- [6] H. D'cotta, A. Fostier, Y. Guiguen, M. Govoroun, J. F. Baroiller, Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia *Oreochromis niloticus*, *Mol. Reprod.* 59 (2001) 265-276.
- [7] C. F. Chang, B. Y. Lin, Estradiol-17 β stimulates aromatase activity and reversible sex change in protandrous black pogy, *Acanthopagrus schlegeli*, *J. Exp. Zool.* 280 (1998) 165-173.
- [8] R. H. Devlin, Y. Nagahama, Sex determination and sex differentiation in fish: An overview of genetic, physiological and environmental influences, *Aquaculture* 208 (2002) 191-364.
- [9] M. Nakamura, R. K. Bhandari, M. Higa, The role estrogen play in sex differentiation

and sex change of fish, *Fish. Physiol. Biochem.* 28 (2003) 113-117.

[10] T. Sunobe, M. Nakamura, Y. Kobayashi, T. Kobayashi, Y. Nagahama, Gonadal structure and P450_{scc} and 3 β -HSD immunoreactivity in the gobiid fish *Trimma okinawae* during bidirectional sex change, *Ichthyol. Res.* 52 (2005) 27-32.

[11] T. Sunobe, M. Nakamura, Y. Kobayashi, T. Kobayashi, Y. Nagahama, Aromatase immunoreactivity and the role of enzyme in steroid pathways for inducing sex change in the hermaphrodite gobiid fish *Trimma okinawae*, *Comp. Biochem. Phys. B.* *in press*.

[12] K. Morohashi, S. Honda, Y. Inomata, H. Handa, T. Omura, A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s, *J. Biol. Chem.* 267 (1992) 17913-17919.

[13] D. S. Lala, D. A. Rice, K. L. Parker, Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of *fushu tarazu-factor I*, *Mol. Endocrinol.* 6 (1992) 1294-1258.

[14] S. Honda, K. Morohashi, M. Nomura, H. Takeya, M. Kitajima, T. Omura, Ad4BP regulating steroidogenic P-450 gene is a member of steroid receptor superfamily, *J. Biol. Chem.* 268 (1993) 7494-7502.

[15] K. Morohashi, T. Omura, Tissue specific transcription of P450 (11 β) gene in vitro, *J. Biol. Chem.* 108 (1990) 1050-1056.

[16] M. Watanabe, M. Tanaka, D. Kobayashi, Y. Yoshiura, Y. oba, Y. Nagahama, Medaka (*Oryzias latipes*) FTZ-F1 potentially regulates the transcription of P450 aromatase in ovarian follicles: cDNA cloning and functional characterization, *Mol. Cell. Endocrinol.* 149 (1999) 221-228.

[17] M. Ito, A. Matsuda, K. Yumoto, A. Takahashi, N. Takamatsu, H. Kanda, S. Yamashita, T. Shiba, cDNA cloning of a new member of FTZ-F1 subfamily from a

rainbow trout, *Biochem. Biophys. Acta.* 1395 (1998) 271-274.

[18] D. Liu, Y. LeDrean, M Ekker, F. Xiong, C. L. Hew, Teleost FTZ-F1 homolog and its splicing variant determine the expression of the salmon gonadotropin II beta subunit gene, *Mol. Endocrinol.* 11 (1997) 877-890.

[19] H. Ellinger-Ziegelbauer, A. K. Hishi, V. Laudet, H. Keller, W. Wahli, C. Dreyer, FTZ-F1-related orphan receptors in *Xenopus laevis*: Transcriptional regulators differentially expressed during early embryogenesis, *Mol. Cell. Biol.* 14 (1994) 2786-2797.

[20] T. Kudo, S. Sutou, Molecular cloning of chicken FTZ-F1-related orphan receptors, *Gene* 197 (1997) 261-268.

[21] Y. Yoshiura, B. Senthilkumaran, M. watanabe, Y. Oba, T. Kobayashi, Y. Nagahama, Synergistic expression of Ad4BP/SF-1 and cytochrome P-450 aromatase (ovarian type) in the ovary of Nile tilapia, *Oreochromis niloticus*, during vitellogenesis suggests transcriptional interaction, *Biol. Reprod.* 68 (2003) 1545-1553.

[22] W. Zhang, X. Li, Y. Zhang, L. Zhang, J. Tian, G. Ma, cDNA cloning and mRNA expression of a FTZ-F1 homologue from the pituitary of the orange-spotted grouper, *Epinephelus coioides*, *J. Exp. Zool.* 301A (2004) 691-699.

[23] M. Higa, H. Ando, A. Urano, Expression of *Fushi tarazu* factor 1 homolog and pit-1 genes in the pituitaries of pre-spawning chum and sockeye salmon, *Comp. Biochem. Physiol. B.* 129 (2001) 503-509.

[24] X. Luo, Y. Ikeda, K. L. Parker, A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation, *Cell* 77 (1994) 481-490.

[25] Y. Sadovsky, P. A. Crawford, K. G. Woodson, J. A. Polish, M. A. Clements, L. M. Tourtellotte, K. Simburger, J. Milbrandt, Mice deficient in the orphan receptor

steroidogenic factor I lack adrenal glands and gonads but express P-450 side chain cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids, *Proc. Natl. Acad. Sci. USA.* 92 (1995) 10939-10943.

[26] J. W. Clemens, D. S. Lala, K. L. Parler, J. S. Richards, Steroidogenic factor-1 binding and transcriptional activity of the cholesterol side-chain cleavage promoter in rat granulosa cells, *Endocrinology* 134 (1994) 1499-1508.

[27] S. Leers-Sucheta, K. Morohashi, J. Mason, M. Melner, Synergistic activation of the human type 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase promoter by the transcription factor steroidogenic factor-1/adrenal 4-binding protein and phorbol ester, *J. Biol. Chem.* 272 (1997) 7960-7967.

[28] P. J. Lynch, S. D. Lala, J. J. Peluso, W. Luo, L. K. Parker, A. B. White, Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues, *Mol. Endocrinol.* 7 (1993) 776-786.

[29] M. S. Grober, T. Sunobe, Serial adult sex change involves rapid and reversible changes in forebrain neurochemistry, *Neuro. Report.* 7 (1996) 2945-2949.

[30] Y. Kobayashi, T. Kobayashi, M. Nakamura, T. Sunobe, C. E. Morrey, N. Suzuki, Y. Nagahama, Characterization of two types of P450 aromatase in the serial-sex changing gobiid fish, *Trimma okinawae*, *Zool. Sci.* 21 (2004) 417-425.

[31] C. A. Heid, J. Stevens, K. J. Livak, P. M. Williams, Real time quantitative PCR, *Genome Res.* 6 (1996) 986-994.

[32] L. H. Wang, S. Y. Tsai, R. G. Cook, W. G. Beattie, M. J. Tsai, B. W. O'Malley, Coup transcription factor is a member of the steroid receptor superfamily, *Nature* 340 (1989) 163-166.

[33] P. A. Crawford, J. A. Polish, G. Ganpule, Y. Sadovsky, The activation function-2

hexamer of steroidogenic factor-1 is required, but not sufficient for potentiation by SRC-1, *Mol. Endocrinol.* 11 (1997) 1626-1635.

[34] H. Ueda, G. C. Sun, T. Murata, S. Hirose, A novel DNA-binding motif abuts the zinc finger domain of insect nuclear hormone receptor FTZ-F1 and mouse embryonal long terminal repeat-binding protein, *Mol. Cell. Biol.* 12 (1992) 5667-5672.

[35] L. Galarneau, J. F. Pare, D. Allard, D. Hamel, L. Levesque, J. D. Tugwood, S. Green, L. Belanger, The alpha-fetoprotein locus is activated by a nuclear receptor of the *Drosophila* FTZ-F1 family, *Mol. Cell. Biol.* 16 (1996) 3853-3865.

[36] D. L. Carlone, J. S. Richards, Functional interactions, phosphorylation, and levels of 3', 5'-cyclic adenosine monophosphate- regulated and constitutive expression of aromatase in gonadal cells, *Mol. Endocrinol.* 11 (1997) 292-304.

[37] P. Zhang, S. H. Mellon, The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3', 5'-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17 alpha-hydroxylase/c17-20 lyase), *Mol. Endocrinol.* 10 (1996) 147-158.

[38] S. L. Fitzpatrick, D. L. Carlone, R. L. Robker, J. S. Richards, Expression of aromatase in the ovary: down regulation of mRNA by the ovulatory luteinizing hormone surge, *Steroid* 62 (1997) 197-206.

Figure legend

Fig. 1. Nucleotide and deduced amino acid sequence of goby *Ad4BP/SF-1*. The numbers on the right refer to the position of the nucleotide. Arrows indicate the target sequences of primers used for cloning and real time quantitative RT-PCR. (see Material and Methods for isolation of goby *Ad4BP/SF-1* cDNA).

Fig.2. Alignment of the amino acid sequences of the *Ad4BP/SF-1* of goby with that of tilapia, medaka, zebrafish, goldfish, catfish, *Xenopus*, chicken, bovine and mouse. Asterisks and dots denote the identical and similar amino acid residues, respectively. Gaps were introduced to improve alignment (-). To aid comparisons, box including the DNA-binding domain, the *Ad4BP/SF-1* box, regions II and III and the AF-2 motif indicate functional domains and motifs. For GenBank accession numbers, refer to Material and methods.

Fig. 3. RT-PCR analysis of the tissue distribution patterns of goby *Ad4BP/SF-1* and *β-actin*. The positive control (PC) consists of a product amplified from the plasmid DNA of goby *Ad4BP/SF-1* and *β-actin*; the negative control (NC) contained no cDNA template.

Fig. 4. Transcripts of *Ad4BP/SF-1* determined using real time quantitative RT-PCR in ovary (black-column) and testis (white-column) of female and male phase individuals (These fishes are the one day prior spawning; see Fig. 5). The numbers in the parentheses are the sample size. Different letter represents the significant difference

($p < 0.05$). Results are the mean $\pm$ SEM.

Fig. 5. Expression of Ad4BP/SF-1 transcripts in ovary during the spawning cycle. Abundance of transcripts was measured by real-time quantitative RT-PCR. The numbers in the parentheses are the sample size. A Kruskal-Wallis test was used to determine significant difference. Groups sharing the same letter codes were not significantly different at $P < 0.05$. The data are the mean $\pm$ SEM.

Fig. 6. Changes in the expression of Ad4BP/SF-1 genes in the goby ovary during sex change from female to male (**a**) and male to female (**b**). Abundance of goby Ad4BP/SF-1 transcripts as a measure of gene expression was determined by real-time quantitative RT-PCR. The numbers in the parentheses are the sample size. The data are the mean $\pm$ SEM. A student *t*-test test was used to determine significant difference. Asterisk (*) denotes significant ($P < 0.05$), in comparison with the preceding groups.

Fig. 1
Yasuhisa Kobayashi

GTT CTC ATC AGG TGT GTT TGT GTC GTG ACA GGC TTT TTT CCC TCC CCT TTT ATT ACC GAG	60								
ACA CAG AAC GAG CTG ACT GAC CGT GTA CAG CCC GGA CCG GAC TTT TTT TAA AAA CCG TCC	120								
3GSP3									
AAA GCC CGA AAA GCA GAG GCC CAT GAG AGG CTG AGG GGG GTC TGT CTC TTC CAG GTT CAC	180								
GAC TAT CAC CAC CGC TGC TGC TGC TCG ATG CTG GGA GAC AAG ACT CAC GGT GTG ACT CTG	240								
M L G D K T H G V T L									
dn sence									
AAG GTT ATG GAG TAC ACG TAC GAC GAC GAC CTG GAG GAG CTG TGT CCG GTC TGT GGA GAC	300								
K V M E Y T Y D D D L E E L C P V C G D									
AAA GTG TCC GGG TAC CAC TAC GGC CTG CTC ACC TGC GAG AGC TGC AAG GGT TTC TTC AAG	360								
K V S G Y H Y G L L T C E S C K G F F K									
3GSP1									
AGG ACA GTA CAG AAT AAT AAA CGA TAC ACG TGT GCG GAG AAC CAG GAG TGT AAA ATA GAC	420								
R T V Q N N K R Y T C A E N Q E C K I D									
3GSP2									
5GSP2									
AAG ACT CAG CGG AAG AGG TGT CCG TTC TGT CGG TTC CAG AAA TGT CTC AAC GTC GGG ATG	480								
K T Q R K R C P F C R F Q K C L N V G M									
5GSP1									
CGC CTG GAA GCG GTG CGT GCA GAC CGA ATG CGT GGG GGG AGG AAT AAA TTC GGC CCG ATG	540								
R L E A V R A D R M R G G R N K F G P M									
dn antisense									
TAC AAG CGG GAC AGG GCC TTG AAG CAG CAG AAG AAA GCC TTG ATA CGC TCC AAC GGT TTC	600								
Y K R D R A L K Q Q K K A L I R S N G F									
AAG CTG GAG AGT ACG GCA GTG TCC GCC CCG CCG CCC GCC TCT CCC CTG CAG GCC GAC TAC	660								
K L E S T A V S A P P P A S P L Q A D Y									
ACC TTC AGC ACG GCC CTG CAC ACC CTG CCC ACC ATC TCC AAG TCC CTG CTG CCC TCC ACC	720								
T F S T A L H T L P T I S K S L L P S T									
ACC CCC AGC TCC CTG CCC CCC ACA GAC TAC GAG GCC AAC CTG TAC GGG CCC TCG CTG GGC	780								
T P S S L P P T D Y E A N L Y G P S L G									
ATG GCC ATG CAG TCC CAC GTG TCC CTG AGC ACC CAG TAC CAG TAC ACC GCC TTC CCC AGC	840								
M A M Q S H V S L S T Q Y Q Y T A F P S									
AGA GCC ATC AAA GCG GAG TGC CCC GAC TAC ACC AGC TCC CCG GAC TCC CTC ACC GGA TAC	900								
R A I K A E C P D Y T S S P D S L T G Y									
CCC TAC CCA GAC ATG TAC CCC TCT TTG TCC CCG CAG CCC CCC AGC CTG CCC CCC CTG GTG	960								
P Y P D M Y P S L S P Q P P S L P P L V									
CTG GAG CTG CTG CGC TGC GAC CCG GAC GAG CTG GTG GTG CAG AAC AAG ATC GTG GCC CAC	1020								
L E L L R C D D E L V V Q N K I V A H									
CTG CAG CAG GAG CAG GGC GGC CGG GGC AGA CTG GAC AAG CCC AGC ACC TTC AGC CTC ATG	1080								
L Q Q E Q G G R G R L D K P S T F S L M									
TGC CGC ATG GCC GAC CAG ACG CTC TTC TCC ATC GTG GAG TGG GCG CGC AGC TGC GTC TTC	1140								
C R M A D Q T L F S I V E W A R S C V F									
TTC AAG GAG CTC AGG GTG GGG GAC CAA ATG AAG CTT CTC CAC AAC TGC TGG TCT GAG CTC	1200								
F K E L R V G D Q M K L L H N C W S E L									
CTG GTT TTG GAT CAT ATT TTC AGA CAA GTG CAG CAC ACG AAG GAA GAC AGT ATC CTG CTA	1260								
L V L D H I F R Q V Q H T K E D S I L L									
GTC ACG GGC CAA GAG GTG GAG CTG TCG TCC CTG CTG TCC CAG GCC GAG GCC ACT CTG TCC	1320								
V T G Q E V E L S S L L S Q A E A T L S									
AGT CTG GTG CAG CGA GGG CAG GAG CAG GCG GCG AGG CTG CGG GCG CTG CAG GTC GAC CGC	1380								
S L V Q R G Q E Q A A R L R A L Q V D R									
rtq F1									
AGG GAG ATC GCC TGC CTC AAG TTC ATG CTC CTC TTC AAC CCC AAT GTG AAG CTG CTG GAG	1440								
R E I A C L K F M L L F N P N V K L L E									
rtq R1									
AAC CAG GCG TTC GTG GAG GGA GTG CAG GAG CAG GTG AAC GGG GCG CTG CTG GAG TAC ACC	1500								
N Q A F V E G V Q E Q V N G A L L E Y T									
GTG TCG TCG TAC CCT CAG TTC CAG GAG AAG TTC AGT CAG CTG CTG CTG CGG CTG CCG GAG	1560								
V S S Y P Q F Q E K F S Q L L L R L P E									
CTG CGC GCC CTC AGC ACT CAG GCT GAG GAC TAT CTG TGC TAC ATG CAC CTC AAC GGA GAG	1620								
L R A L S T Q A E D Y L C Y M H L N G E									
GTG CCC TGC AAC AAC CTG CTC ATC GAG ATG CTG CAC GCC AAG CGC GCC TGC GTC TGA GGA	1680								
V P C N L I E M L H A K R A C V ***									
5GSP3									
CTG CTT CTT TGT ATC TTT GTA TGA AAG TGC ACA TGA GAC TAA ACA ATC AAC TAA ACT AAA	1740								
CTA AAC GGT CCT TTA TTC ACA CCT GCC CGG GCG GCC GCT CGA GCC CTA TAG TGA GT	1797								

DNA binding domain

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goby          -----MLGDKTHGVTLLKVM EYTYDDLEELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKRYTCAENQECKIDKTQRKRCFP
tilapia       -----MLGDKAHGVTLTKVMEYTYDELDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKRYTCAENQECKIDKTQRKRCFP
medaka        -----MLGDKAHGVTLTKVMEYTYDELDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKRYTCAENQECKIDKTQRKRCFP
zebrafish     MLPKVESEYLGRLARSHGEGQHMPGNMQAPQPKMMDYSYDELDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKRYTCAENQSCQIDKTQRKRCFP
goldfish      -----MDYSYDTELDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKRYTCAESQDCKIDKTQRKRCFP
catfish       -----MEFTTEEDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKRYTCAENQDCGIDKTQRKRCFP
Xenopus       MLPKVESEALGLSLRHGEGQHMPDNMQVSQYKMMGYSYDELDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKRYTCAENQSCQIDKTQRKRCFP
chicken       -----MDYSYDELDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKHYTCTESQNKCIDKTQRKCCFP
bovine        -----MDYSYDELDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKHYTCTESQSKCIDKTQRKRCFP
mouse         -----MDYSYDELDLELCPVCGDKVSGYHYGLLTCESCKGFFKRTVQNNKHYTCTESQSKCIDKTQRKRCFP

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Ad4BP/SF-1 box

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goby          CRFQKCLNVGMRLEAVRADMRGGRNKFGPMYKRDRAIKQKKALIRNSNGFKLEST---AVSAPPASPLQADYFTSTALHTLPTISK-----SLLPS
tilapia       CRFQKCLNVGMRLEAVRADMRGGRNKFGPMYKRDRAIKQKKALIRNSNGFKLES-----TAPPASPLQADYFTGLTLHTLPTISK-----SLLPS
medaka        CRFQKCLNVGMRLEAVRADMRGGRNKFGPMYKRDRAIKQKKALIRNSNGFKLES-----TALPSASPLQADYFTGLTLHTLPTISK-----SLLPS
zebrafish     CRFQKCLTVGMKLEAVRADMRGGRNKFGPMYKRDRAIKQKKALIRANGLKLEAMTVQMVTVPADLTTISAIGNIHASAKGLPLSHHHHHHHHHHHHHSS
goldfish      CRFQKCLNVGMRLEAVRADMRGGRNKFGPMYKRDRAIKQKKALIRASGLKMEAT-----PPLMSPSQSDYSFSTALSTPAPKNT-----HLNIG
catfish       CRFQKCLSVGMRLEAVRADMRGGRNKFGPMYKRDRAIKQKKRALIRASTFKLEHN-----PLVPSNQADSEYFPF-----GSVGP
Xenopus       CRFQKCLTVGMKLEAVRADMRGGRNKFGPMYKRDRAIKQKKALIRANGLKLEAIVQIVISITPDLTSSAIGNIHASAKGLHLN-----GSVGP
chicken       CRFQKCLTVGMRLEAVRADMRGGRNKFGPMYKRDRAIKQKKALIRANGFKLETGVPQVPSVQNDYGLSSTIHSIHAMAKTLPPN-----
bovine        CRFQKCLTVGMRLEAVRADMRGGRNKFGPMYKRDRAIKQKKAQIRANGFKLETGPPVGVPPPPPP---PPDYMLPHGLHASEPK-----
mouse         CRFQKCLTVGMRLEAVRADMRGGRNKFGPMYKRDRAIKQKKAQIRANGFKLETGPPMGVPPPPPP---PPDYMLPPSLHAPEPK-----
*****.***:*****.*****.***:*  ***.:*:

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goby	TPPSSLPTDYEANLYGPSLGMAMQSHVSLSTQYQYT--AFPSRAIKAECPD-----YTSSPDSLTGYPPDMPYSLSFPQP-----PSLPPLVLVEL
tilapia	TPSSIPTDYEANLYGPPSLGMAMQSHVPLTTQYQYT--AFPGRAIKAECPD-----YTSSPETLTGYPPDDMYPSPASFPQ-----PSLPPLVLVEL
medaka	TTSVPPTDYEANLYGPPSLGMAMQSHVLNPNQYQYT--AFPSRAIKAECPD-----YTSSPELTGYPPDPVPSASFPQ-----PSLPPLVLVEL
zebrafish	SSAGLPADFDRLSPFVTSPVSMAMPFHAGGLQGYYAQYG-HFQSRTIKSEYPTTQTAR-----QSPHGLPLRRSLRSGSFPSS-----FPHVLVLVEL
goldfish	TSVAPTDYERNLYAGSSSLTLTIPIPAHTLPLAQQPAYPD-TLPSRAIKSEYPDHYTSEHYTSASSPESITPGTYVDQTVSTPSLAPLGLTVPLVLVEE
catfish	LLAPLGPPDYPDCPPACPPSLGLVALHSYGSFFPAQYQYTTPTVPGRSIKAEHPDP-----YSGSPDSSLGYSYAEGCIAASPQTSPLN-PAVPSLVLEL
Xenopus	-HAALPPSDYDRSPFVTSPISMAMPHG-SLQGYQSYG-HFPSRAIKSEYPDDPYTSSP-----ESIIMGVSMDMSYQSSSPSN-----I PHIVLEL
chicken	-PAALTPADYERGPYGTSPMLATVPG-HTPLAGYHP--SFNNRAIKSEYPDHYSAAH-----EAVPTYAYPETYPSSSPPD-----IPHELVL
bovine	-GLAGGGPAGPLGDGFAPALPMAPVASHCPLAGYLYP--AFPGRAIKSEYPEFYASPP-----QQ--PGPPYGYPEPFSGGPG-----VPELILQL
mouse	-ALVSGPPSGPLGDGFAPS LPMAPVGPHGHLAGYLYP--AFSNRTIKSEYPEFYASPP-----QQ-PGPPYSYPEPFSGGN-----VPELILQL

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: : * * * * *
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Region II
: * :::

Region II

[illegible]

Region II

[illegible]

AF-2

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goby      QEKFSQLLRLRPELRALSTQAEADLYCYMHLNGEVPCCNNLLIEMLHAKRACV-
tilapia  QEKFSQLLVRLPELRSLSTQAEADLYCYMHVSGEVPCNNLLIEMLHAKRACV-
medaka    QEKFSQLLVRLPELRSLSTQAEADLYCYMHLNGEVPCCNNLLIEMLHAKRACV-
zebrafish TDKFGQLLRLRPELRALSTQAEELYKYHKLNGDVPCCNNLLIEMLHAKRA----
goldfish  PDRFSQILLRLPELRALSTQAEADLYCYKHLNGEVPCCNNLLIEMLHAKRTCI-
catfish   VDRFSQVLRLRPEVRALSAQAEELYSSKHLSGDVPCCNNLLIEMLHAKRATTI-
Xenopus   TDKFGQLLRLRPELRALSTQAEELYKYHKLNGDVPCCNNLLIEMLHAKRA----
chicken   TDKFRQLLRLRTEVRALSMQAEELYHKHLNGEVPCCNNLLIEMLHAKRT----
bovine     GDKFQQLLLCIVEVRALSMQAEELYHKHLGNEMPRNNLLIEMLQAKQT---
mouse     GDKFQQLLLCIVEVRALSMQAEELYHKHLGNEMPRNNLLIEMLQAKQT---

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Fig.3
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Fig. 4
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Fig. 5
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Fig. 6
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