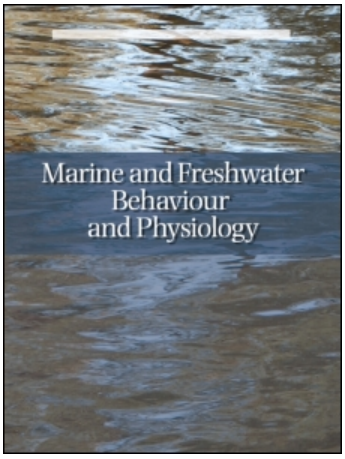

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[a] Aquaculture Industry Division, East Sea Fisheries Research Institute, National Fisheries Research & Development Institute, Gangneung 210-861, Republic of Korea [b] Division of Marine Environment & BioScience, Korea Maritime University, Busan 606-791, Republic of Korea [c] Department of Marine Life Sciences, Jeju National University, Jeju Special Self-Governing Province 690-756, Republic of Korea [d] Division of Electrical & Electronic Engineering, Korea Maritime University, Busan 606-791, Republic of Korea

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Molecular cloning and expression of TRα and TRβ in the protandrous cinnamon clownfish, *Amphiprion melanopus* during sex reversal

Mi Seon Park[a], Cheol Young Choi[b]*, Na Na Kim[b], Kwang Wook An[b], Jehee Lee[c] and Gyung-Suk Kil[d]

[a]*Aquaculture Industry Division, East Sea Fisheries Research Institute, National Fisheries Research & Development Institute, Gangneung 210-861, Republic of Korea;* [b]*Division of Marine Environment & BioScience, Korea Maritime University, Busan 606-791, Republic of Korea;* [c]*Department of Marine Life Sciences, Jeju National University, Jeju Special Self-Governing Province 690-756, Republic of Korea;* [d]*Division of Electrical & Electronic Engineering, Korea Maritime University, Busan 606-791, Republic of Korea*

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We divided the process of sex reversal from immature male to mature female in the protandrous cinnamon clownfish (*Amphiprion melanopus*) into six developmental stages as follows: I, immature male; II, mature male; III, male at 60 days after female removal; IV, male at 90 days after female removal; V, male at 120 days after female removal; and VI, mature female. Thyroid hormone receptors α (TRα) and β (TRβ) cDNAs were cloned from the ovary and mRNA expression levels were compared during the sex-reversal process. The nucleotide sequences of the TRα and TRβ cDNA were 1230 and 1188 bp in length with open reading frames encoding peptides of 409 and 395 amino acids, respectively. We observed that TRα mRNA and protein levels were high in all stages except the immature gonad, while TRβ mRNA levels were higher in the mature ovary than in any other gonadal stage. We injected gonadotropin-releasing hormone analogue to identify its effects on TRs mRNA in immature fish. The mRNA levels of TRs increased significantly. We therefore propose that TRs are related to testicular development as well as ovarian development in cinnamon clownfish. The present study also provides basic data on the role of TRs during sex reversal in fish.

Keywords: sex reversal; cinnamon clownfish; *Amphiprion melanopus*; thyroid hormone receptor; GnRHa

Introduction

The thyroid hormones (THs), namely 3,5,3′-triiodo-L-thyronine (T_3) and thyroxine (T_4) are involved in growth, development, differentiation, and metabolism in vertebrates, including teleost fishes (Shi et al. 1996; Power et al. 2001). The physiological actions of THs are mediated through thyroid hormone receptors (TRs), which belong to the nuclear receptor superfamily (Mangelsdorf et al. 1995) and are ligand-dependent transcription factors (Ribeiro et al. 1998). The ligand-activated receptor binds to specific DNA sequences harbored in gene promoters,

*Corresponding author. Email: choic@hhu.ac.kr

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termed thyroid response elements (TREs), and regulates transcription of target genes (Marchand et al. 2001). The nuclear receptors have conserved domains that are important for their functionality. The DNA-binding domain (DBD) and the ligand-binding domain (LBD) in particular are highly conserved among vertebrate species (Nelson and Habibi 2006). The DBD interacts with nuclear proteins at the TREs and in turn regulates TR gene activation (Harvey and Williams 2002).

There are at least two TR isoforms in vertebrates, including humans (Weinberger et al. 1986), rats (Thompson et al. 1987), chickens (Sap et al. 1986), frogs (Schneider and Galton 1991), and fish (olive flounder, *Paralichthys olivaceus* (Yamano et al. 1994), zebrafish, *Danio rerio* (Liu et al. 2000), Atlantic salmon, *Salmo salar* (Marchand et al. 2001), goldfish, *Carassius auratus* (Nelson and Habibi 2006), and black porgy, *Acanthopagrus schlegeli* (An et al. 2010)).

TRs have been shown to be associated with postnatal avian development, amphibian metamorphosis, and smoltification in fish (Gauthier et al. 1999; Galay-Burgos et al. 2008; Manchado et al. 2009). TRs are present in oocyte nuclei (Maitra and Bhattacharya 1989), and there are changes in TR subtype expression level during different stages of reproductive maturity (Nelson and Habibi 2006, 2009). Furthermore, TRs were found to be higher in mature gonads than in immature gonads in fathead minnow, *Pimephales promelas* (Filby and Tyler 2007), and black porgy, *A. schlegeli* (An et al. 2010). Yamano and Miwa (1998) reported on the ubiquitous and constant expression of TRα mRNA during early developmental stages and on the late stage or tissue-specific expression of TRβ mRNA during metamorphosis in olive flounder. Therefore, it is suggested that TRs are involved in gonadal development and reproduction in fish (Yamano and Miwa 1998; Filby and Tyler 2007).

Cinnamon clownfish, *Amphiprion melanopus*, groups consist of a mated adult pair (functional female and male) and/or an immature individual; their sex is controlled by social rank in the group. The female is the largest and the dominant fish in the group. When the dominant female dies or is absent, the male undergoes sex reversal (Godwin and Thomas 1993; Godwin 1994). Hence, cinnamon clownfish is a good model for the study of the sex change mechanism in protandrous hermaphrodite fish. Thus far, only studies on the changes in steroid hormone (Godwin and Thomas 1993) and histological analysis (Godwin 1994) have been reported during sex change processes in cinnamon clownfish; genetic factors involved in sex change processes have not been investigated.

We isolated the two types of TRs (TRα and TRβ) expressed in the gonad of cinnamon clownfish and investigated the expression pattern of their mRNAs to understand the role of TRs in sex-reversal processes. We also compared the time-course expression of TR mRNA after injection of gonadotropin-releasing hormone analogue (GnRHa) to clarify the role of the TRs.

Materials and methods

Experimental fish

The cinnamon clownfish [immature ($n = 20, 7.1 \pm 0.8$ g); mature male ($n = 20$, 10.5 ± 1.2 g), and mature female ($n = 20, 22.2 \pm 2.1$ g)] were purchased from the Center of Ornamental Reef & Aquarium (CCORA, Jeju, Korea). We placed them in

groups that consisted of a mated pair (dominant female and male) and each group was reared in one of fifteen 50L circulation filter tanks prior to the experiments. The temperature was maintained at $28 \pm 0.5^{\circ}C$, and the photoperiod was a 12 : 12 h light–dark cycle. Fish were fed a commercial marine aquarium fish feed (jeilfeed company, Kyoungnam, Korea) twice a day.

In the present study, the experimental design for sex reversal was modified from the methods of Godwin and Thomas (1993). After the groups consisting of a mated pair (dominant female and male) were made, sex change was induced in male fish by removing the female from each group and adding the immature clownfish. After 60, 90, and 120 days from removing the female, we observed the male fish undergoing the sex change process from male to female. We divided the process of sex reversal into six developmental stages as follows: I, immature male; II, mature male; III, male at 60 days after female removal; IV, male at 90 days after female removal; V, male at 120 days after female removal (mostly ovary); and VI, mature female. The fish were anesthetized in tricaine methane sulfonate (MS-222; Sigma, USA) and decapitated prior to tissue collection. Gonads from the fish at the each gonad maturity stage were removed, immediately frozen in liquid nitrogen, and stored at $-80^{\circ}C$ until the total RNA was extracted for analysis.

cDNA cloning of TRα and TRβ genes

Partial fragments of the TRα and TRβ genes were amplified as follows. Primers for TRα were designed using highly conserved regions of Senegalese sole, *Solea senegalensis* TRαA (GenBank accession no. AB366000) and orange-spotted grouper, *Epinephelus akaara* TRαA (EF502002): TRα forward primer (5′-GAG TGG GAG CTG ATC AGG AT-3′) and TRα reverse primer (5′-CGT CAC CTT CAT CAG CAG CT-3′). Primers for TRβ were designed using highly conserved regions of orange-spotted grouper, *E. akaara* TRβ (EF502004) and black porgy, *A. schlegeli* TRβ (EF605274): TRβ forward primer (5′-AAC CAC TGG AAG CAG AAG CG-3′) and TRβ reverse primer (5′-CAC CTT CAT TAG CAG CTT GG-3′).

Total RNA was extracted from the gonad using a TRIzol kit (Gibco/BRL, USA). Reverse transcription (RT) was conducted using M-MLV reverse transcriptase (Bioneer, Korea), and polymerase chain reaction (PCR) amplification was performed using TaKaRa Taq (Takara, Japan), as described by Choi and Habibi (2003). RT-PCR was subsequently performed for 40 cycles as follows: one cycle of denaturation at $94^{\circ}C$ for 2 min, denaturation at $94^{\circ}C$ for 30 s, annealing at $56^{\circ}C$ for 40 s, and extension at $72^{\circ}C$ for 1 min, followed by one cycle of 7 min at $72^{\circ}C$ for the final extension. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide. The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, USA), used to transform DH5α cells (RBC Life Sciences, Korea), and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification kit (Cosmo, Korea) and TR DNA inserts were verified by restriction digest with *Eco*RI (Fermentas, USA). Insert identity was confirmed by sequencing plasmid DNA using as ABI DNA Sequencer (Applied Biosystems, USA).

Rapid amplification of TRα and TRβ cDNA 3′ and 5′ ends

For the RACE reaction, total RNA was extracted from the gonad using a TRIzol kit (Gibco/BRL, USA). By using 3 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing^{TM} full-length cDNA Premix kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo-$d(T)_{18}$ anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA $T(T)_{18}$-3′) and a CapFishing^{TM} adaptor (Seegene, Korea).

TRα and TRβ specific primers were selected from the PCR product obtained by RT-PCR. For 3′ RACE, the 50 μL PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 mM 3′ RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′) and 1 μL of 10 mM 3′ RACE TRα-specific primer (5′-ATG ACT CCT GCG ATT ACA CGT GTC GTC-3′) and 1 μL of 10 mM 3′ RACE TRβ-specific primer (5′-CTG CCT TGT GAA GAC CAG ATC ATC CTG-3′) and 25 μL SeeAmp Taq Plus master mix (Seegene, Korea). PCR was performed for 40 cycles as follows: one cycle of denaturation at 94°C for 5 min, denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 60 s, followed by one cycle of 5 min at 72°C for the final extension.

For 5′ RACE, the 50 μL PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 mM 5′ RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mM 5′ RACE TRα-specific primer (5′-GAC GAC ACG TGT AAT CGC AGG AGT CAT-3′) and 1 μL of 10 mM 5′ RACE TRβ-specific primer (5′-CAG CAT GAT CTG GTC TTC ACA AGG CAG-3′) and 25 μL SeeAmp Taq Plus master mix (Seegene, Korea). PCR was performed for 40 cycles as follows: one cycle of denaturation at 94°C for 5 min, denaturation at 94°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 60 s, followed by one final extension cycle of 5 min at 72°C. Amplified PCR products were processed by electrophoresis using 1% agarose gels containing ethidium bromide. The transformation and sequencing were the same as described above.

Western blot analysis

Total protein isolated from the gonads during sex change processes in cinnamon clownfish were extracted using protein extraction buffer (5.6 mM Tris, 0.55 mM EDTA, 0.55 mM EGTA, 0.1% SDS, 0.15 mg mL^{-1} PMSF, and 0.15 mg mL^{-1} leupeptin), sonicated, and quantified using the Bradford method (Bio-Rad, USA). Total protein (30 μg) was loaded per lane onto a 4% acrylamide stacking gel and a 12% acrylamide resolving gel. For reference, a protein ladder (Fermentas, USA) was used. Samples were electrophoresed at 80 V through the stacking gel and 150 V through the resolving gel, until the bromophenol blue dye front had run off the gel. The gels were then immediately transferred to a 0.2 μm polyvinylidene diflouride (PVDF) membrane (Bio-Rad) at 85 V for 1.5 h at 4°C. Thereafter, the membranes were blocked with 5% milk in TBS (pH 7.4) for 45 min, followed by washing in TBS. Membranes were incubated with polyclonal rabbit antibody to TR α1 (dilution 1 : 200, ab5621, Abcam, UK), followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (dilution 1 : 2000, Bio-Rad, USA) for 60 min. Bands were detected using the standard ECL as well as the more sensitive ECL systems (ECL Advance; GE Life Sciences, Sweden) and exposure to autoradiography-sensitive film for 5 min.

GnRH treatment

To investigate the roles of TRs in the reproductive physiology of cinnamon clownfish, immature fish ($n = 30$, 7.1 ± 0.8 g) were treated with GnRH, and gonadal expression of TRs was measured. The fish were reared in 300 L circulation filter tanks in the laboratory. They were then anesthetized with tricaine methane sulfonate (MS-222, Sigma, USA) prior to injection. GnRHa (des Gly^{10}-D-Ala^{6}) luteinizing-hormone releasing hormone ethylamide (Sigma, USA) was dissolved in 0.9% physiological saline. Each fish was given an injection of GnRHa (0.1 and 0.5 $\mu g\, g^{-1}$ body weight (BW) at a volume of 10 $\mu L\, g^{-1}$ BW). After injection, the gonads were removed from five fish at each of the following time periods: 0, 6, 12, 24, and 48 h. During the experimental period, water temperature, and photoperiod were maintained at $28 \pm 0.5^{\circ}$C and 12 : 12 h light–dark, respectively.

Quantitative PCR

Quantitative PCR (QPCR) was performed to determine the relative expression of TRα and TRβ mRNA from the gonad of cinnamon clownfish. Primers for QPCR were designed with reference to the known sequences of cinnamon clownfish as follows: TRα forward primer (5′-GAA GAA CCT CCA TCC ATC CTA CTC C-3′), TRα reverse primer (5′-GCA CTT CTT GAA GCG GCA CAG-3′) and TRβ forward primer (5′-GAT CCA GAG AGT GAG ACG CTT ACG-3′), TRβ reverse primer (5′-AAA CGA GGA GAG TGA CAC ACC AAG-3′) and β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG-3′), β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′). PCR amplification was conducted using a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad, USA), according to the manufacturer's instructions. QPCR was carried out as follows: denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s. After the QPCR, the data from three replicate samples were analyzed with analysis software of the system (Bio-Rad) to estimate transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were found to be as follows: β-actin = 94.7%, TRα = 95.1%, and TRβ = 93.0%. All data were expressed as change with respect to the corresponding β-actin-calculated cycle threshold (ΔCt) levels. The calibrated ΔCt value ($\Delta\Delta$Ct) for each sample and internal control (β-actin) was calculated ($\Delta\Delta Ct = 2^{-(\Delta Ct_{sample} - \Delta Ct_{internal\ control})}$. Also, to ensure that the primers amplified a specific product, we performed a melt curve, melting at only one temperature.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA, followed by Tukey's *post hoc* test, were used to compare differences in the data with significance levels set at $p < 0.05$. Values are expressed as mean $\pm$ SD.

Results

Identification of TRα and TRβ

A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone full-length TRs cDNA. The full-length TRα cDNA contained 1230 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 409 amino acids (EU908059), and TRβ contained 1188 nucleotides, including an ORF encoding a protein of 395 amino acids (EU908058). The amino acid sequence of cinnamon clownfish TRα was compared to those deduced from the cDNA of other vertebrates. The amino acid identity of TRα with those of other vertebrates is: 92% with Atlantic halibut, *Hippoglossus hippoglossus* TRα (AF143296), 92% with olive flounder, *P. olivaceus* TRα (D16461), 91% with orange-spotted grouper, *E. akaara* TRα (EF502002), and 83% with human TRα (M24748). Two conserved domains are located in the cinnamon clownfish TRα: DBD (residues 52–119) and LBD (residues 187–373). The TRα was much conserved in the DBD (93–99%) and LBD (91–94%) compared to other vertebrates (Figure 1). The amino acid identity of TRβ with those of other vertebrates is: 97% with orange-spotted grouper TRβ (EF502004), 96% with black porgy, *A. schlegeli* TRβ (EF605274), 96% with olive flounder, *P. olivaceus* TRβ (D45245), and 85% with human TRβ (X04707). The TRβ also has two conserved domains: DBD (residues 33–99) and LBD (residues 183–374) and shows much conservation in the DBD (94–99%) and LBD (88–98%) compared to that in other vertebrates (Figure 2).

Compared to human TRα1 and TRβ1, cinnamon clownfish TRα and TRβ amino acid sequences had 83% and 85% identity, respectively. Cinnamon clownfish TRs show a high percentage of conservation in the DBD (93–94%) and LBD (88–89%) when compared to human TRs (Figure 3). The DBD that interacts with TREs and the "T and A boxes" that are immediately adjacent to the DBD play an important part in DNA-binding dimerization and forms a long α-helix (Marchand et al. 2001); both "DBD" and "T and A boxes" are very well-conserved in the TRα and TRβ of cinnamon clownfish. In the LBD, helices that form a long α-helix are well-conserved for all sequences examined. Furthermore, the α3, α5, α6, and the two C-terminal helices α11 and α12 are well-conserved in cinnamon clownfish. At the extreme C-terminal, the autonomous transcription-activating region (AF2–AD) is strongly conserved. In cinnamon clownfish TRα, these correspond to Arg^{227}, Arg^{261}, Arg^{265}, and Ser^{276}, and in cinnamon clownfish TRβ, these correspond to Arg^{216}, Arg^{250}, Arg^{254}, and Asn^{265}. These Arg residues are conserved in TRα and TRβ from all known vertebrates, and it is known that these amino acids interact directly with the THs (Marchand et al. 2001). In cinnamon clownfish, the Ser^{276} residue in TRα is substituted by a Ser^{277} residue in human TRα and the Asn^{265} residue in TRβ is conserved in vertebrates (Marchand et al. 2001).

TRβ in fish contains a nine amino acid insertion compared to the other known mammal or amphibian TRβs (Yamano and Inui 1995). These amino acids are presented between α2 and α3 in a region forming a loop in the three-dimensional structure. In fish species, this insertion is varied: cinnamon clownfish (SAAGVKEAK), gilthead seabream, *Sparus aurata* (SAAWVKETK), orange-spotted grouper, *E. akaara* (SAAGVKETK), black porgy, *A. schlegeli* (SAAGVKETK), and Senegalese sole, *S. senegalensis* (SAAGVKEAK).

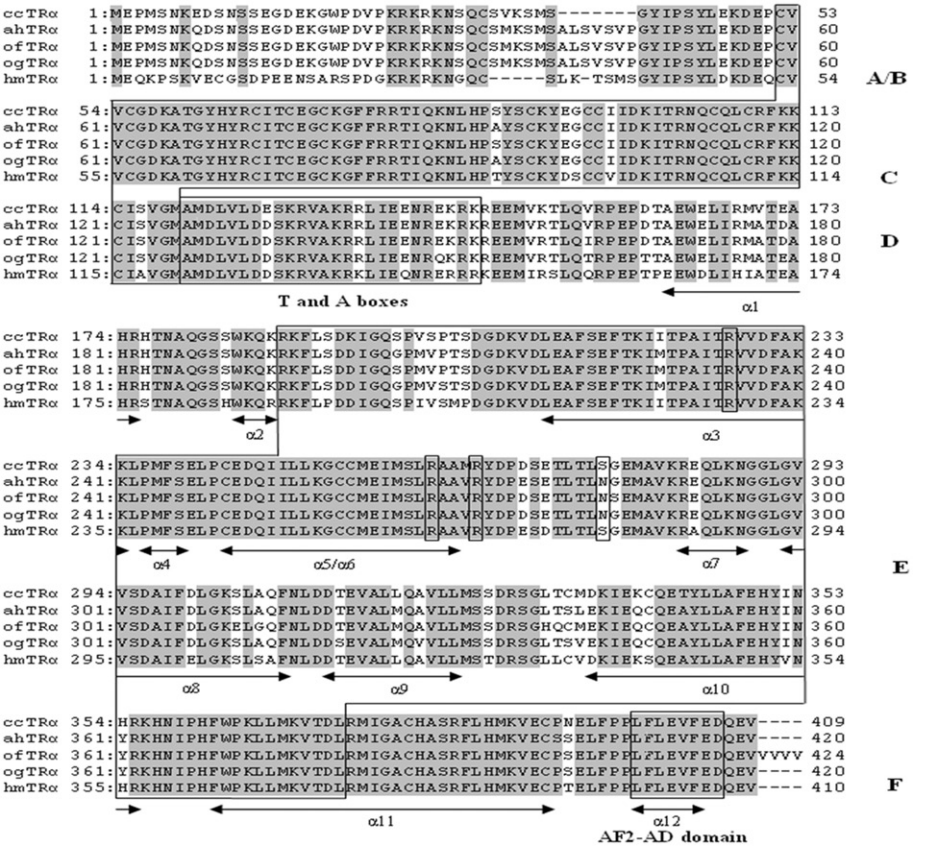

Figure 1. Alignment of the amino acid sequences from the cinnamon clownfish TR α (ccTRα) isolated in this study compared with known TRα from other vertebrates: Atlantic halibut TRα (ahTRα), olive flounder TRα (ofTRα), orange-spotted grouper TRα (ogTRα), and human TRα (hmTRα). Shades indicate amino acids identical to the first line, which correspond to the ccTRα. Dashes indicate gaps inserted to enhance sequence similarity. The limits of the DBD, LBD, T, and A boxes and AF2-AD domain are indicated by boxes. The positions of the helices found in the three-dimensional structure are underlined. The amino acids known to be directly in contact with the ligand in the three-dimensional structure are indicated by boxes.

Expression of TRα and TRβ during sex reversal

The high expression of TRα mRNA was maintained in all gonad developmental stages except immature gonad during sex-change processes (Figure 4B). The increased expression of TRα in the gonads was confirmed in western blot analysis, which revealed a protein with TRα immunoreactivity of a size corresponding to the predicted size for cinnamon clownfish TRα (47 kDa). This amount of protein was similar to the mRNA expression in cinnamon clownfish gonads (Figure 4A). In contrast, the expression of TRβ mRNA was significantly higher in mature ovarian tissue (Figure 4C).

Time-course expression of TRα and TRβ by GnRHa treatment

GnRHa injection increased the expression of TRα and TRβ mRNAs in the immature gonads, as shown in Figure 5. Both TRα and TRβ were highest at

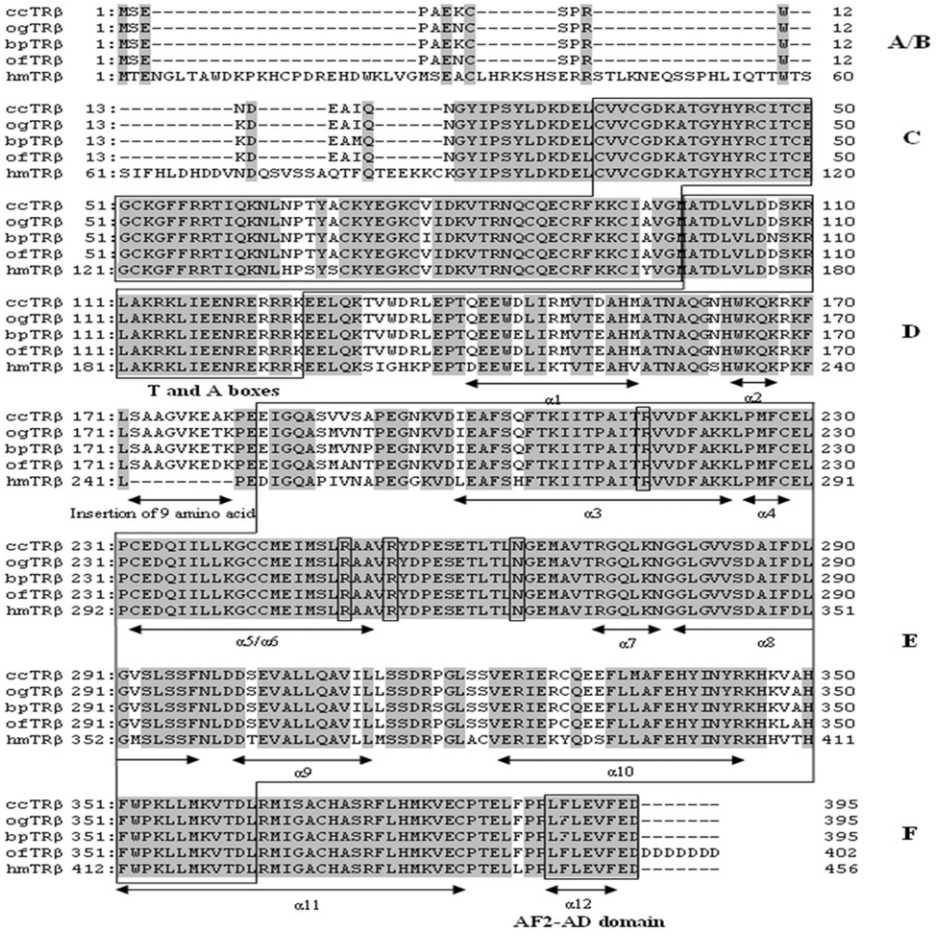

Figure 2. Alignment of the amino acid sequences from the cinnamon clownfish TR β (ccTRβ) isolated in this study compared with known TRβ from other vertebrates: orange-spotted grouper TRβ (ogTRβ), black porgy TRβ (bpTRβ), olive flounder TRβ (ofTRβ), and human TRβ (hmTRβ). Shades indicate amino acids identical to the first line, which correspond to the ccTRβ. Dashes indicate gaps inserted to enhance sequence similarity. The limits of the DBD, LBD, T, and A boxes and AF2-AD domain are indicated by boxes. The positions of the helices found in the three-dimensional structure are underlined. The amino acids known to be directly in contact with the ligand in the three-dimensional structure are indicated by boxes. The teleost-specific nine amino acid insertion in TRβ is underlined.

6 h (at 0.1 $\mu g\,g^{-1}$ GnRHa) and 12 h (at 0.5 $\mu g\,g^{-1}$ GnRHa), but TRβ decreased after 12 h.

Discussion

The process of sex change has been reported for some protandrous fishes: the wrasse, *Halichoeres tenuispinis* (Choi et al. 2005), the gilthead sea bream, *Sparus aurata* (Soverchia et al. 2007), the black porgy, *A. schlegeli* (Wu et al. 2005; An et al. 2008), and the red-spotted grouper, *E. akaara* (Li et al. 2009).

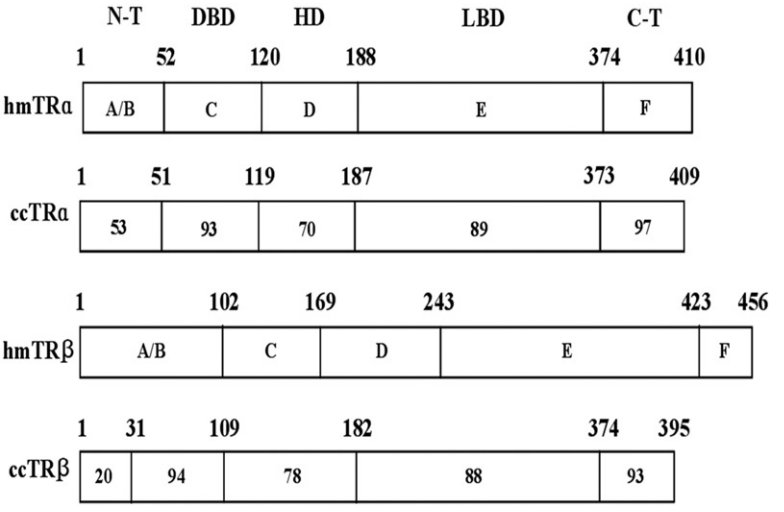

Figure 3. The structures of the five functional domains of human TRs: hmTRα (M24748), hmTRβ (X04707), and cinnamon clownfish TRs: ccTRα (EU908059), ccTRβ (EU908058) are shown. Numbering represents the position of the amino acid that separates four functional domains of the TRα and TRβ. The percentage of amino acid identity of each domain relative to the human TRs is indicated within the box representing the corresponding domain.

The evidence for sex change in these fishes includes: skewed size and age frequency. However, in the case of cinnamon clownfish, they usually live in groups consisting of a mated adult pair and an immature individual, and sex is controlled by social rank in the group. When the largest individual, always female, disappears, the male changes its sex to female (Godwin and Thomas 1993; Godwin 1994). Despite many ecological studies and some physiological studies of sex change in the anemone fishes, physiological mechanisms of sex changing have not yet been clarified (Godwin and Thomas 1993; Godwin 1994; Miura et al. 2008).

In the present study, we cloned two TR genes (TRα and TRβ) from cinnamon clownfish gonad. Like in other vertebrates, the cinnamon clownfish TR sequences have five conserved domains: the N-terminal (N-T) region; the highly conserved DBD, which binds the TRE; the hinge domain (HD), which facilitates the formation of a crucial long α-helix; the LBD, which enables ligand binding; and the C-terminal (C-T) domain, which is important for transcriptional activation (Duncan et al. 2003; Figure 3). As found in other fish (Nelson and Habibi 2009), the cinnamon clownfish TR DBD is highly conserved. The HD is conserved and adjacent to the DBD and plays an important role in DNA binding, in dimerization, and in forming a long α-helix (Marchand et al. 2001). In the LBD, the amino acids that interact directly with the THs are well-conserved in vertebrates. Also, at the C terminus of the C-T domain, AF2-AD is well-conserved and regulates transcription (Marchand et al. 2001). On the other hand, the insertion of nine amino acids (172–180 residues) between helices α2 and α3 in the teleost TRβ is distinctly different. These amino acids have never been identified in any other vertebrates (Marchand et al. 2001; Figure 2). Yamano and Inui (1995) reported that these amino acids appear to be teleost specific. In the cinnamon clownfish, these amino acids were located at residues 172–180 (SAAGVKEAK).

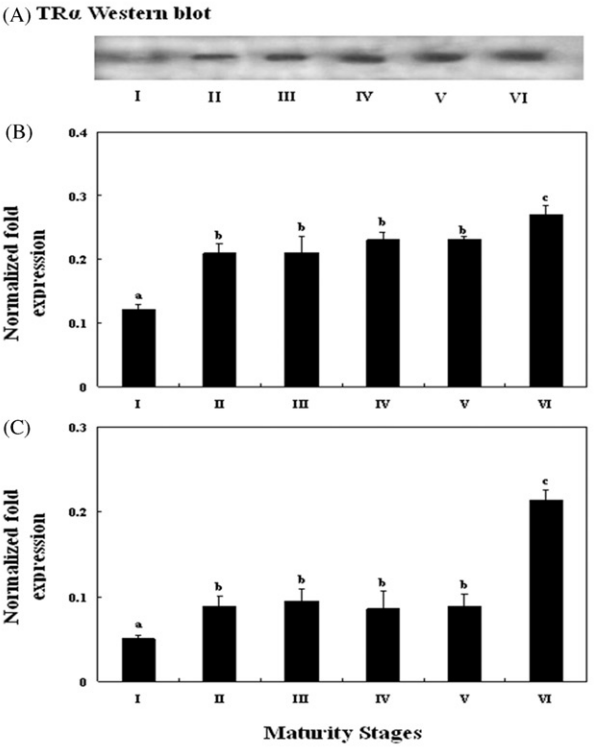

Figure 4. (A) Western blot of TRα protein expression at the each gonad maturity stage during sex-reversal from immature male to female ovary (I, immature male; II, mature male; III, male at 60 days after female removal; IV, male at 90 days after female removal; V, male at 120 days after female removal (mostly ovary); and VI, mature female) in cinnamon clownfish. (B) TRα and (C) TRβ mRNA levels relative to β-actin mRNA levels are analyzed by real-time PCR in the gonad of cinnamon clownfish during sex changes. Values with dissimilar letters are significantly different ($p < 0.05$) from each other. Values are expressed as mean $\pm$ SD ($n = 5$).

We observed the change in TRα and TRβ mRNA expression during sex reversal from male to female and found TRα were significantly high in the mature female, while the expression levels were similar in other developmental stages. TRβ was significantly high only in the mature female. The TRα protein level was high in all gonads, except those of immature fish, in western blot analysis, as in the mRNA expression (Figure 4). Filby and Tyler (2007) reported that TRα was high in all developmental stages and was involved in the development of the testis and ovary. In contrast to TRα, TRβ was high in mature females. These results corresponded to those of a previous study that showed that the expression of TRβ mRNA was high during the sex change process in black porgy, *A. schlegeli* (An et al. 2010); we therefore determined that TRβ is involved in ovary development. In cinnamon clownfish, while the expression of TRα mRNA was constant in the mature stages, TRβ was high in the ovary. TRα appears to play a role in development and maturity, while TRβ has a specific role in ovarian development. Since TRβ was found to increase at specific stages in olive flounder, *P. olivaceus* (Yamano and Miwa 1998), we assume that TRβ has a specific role in the mature female ovary. In addition,

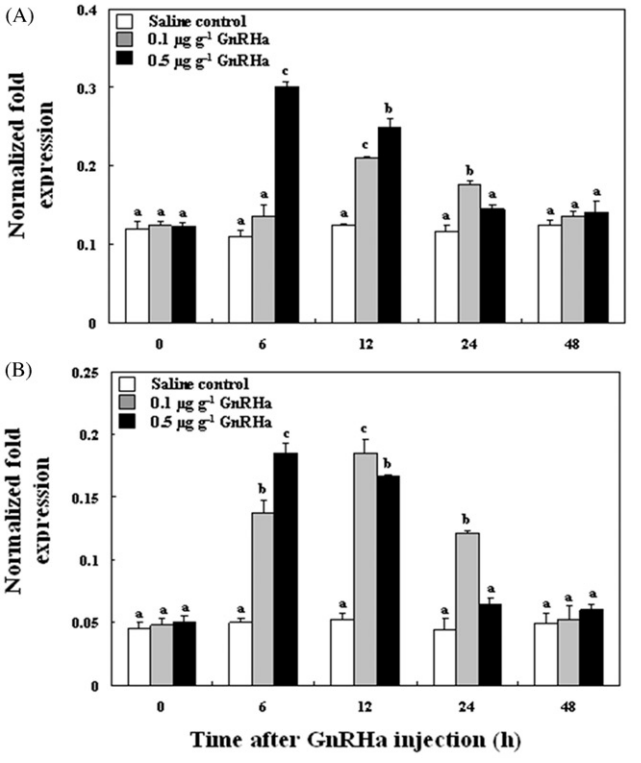

Figure 5. Time-related effect of GnRHa (0.1 and 0.5 μg g^{-1}) on (A) TRα and (B) TRβ mRNA levels in the cinnamon clownfish gonad. Total RNA was extracted 0, 6, 12, 24, and 48 h after treatment, and 3 μg was used for PCR. The expression level of each sample was normalized with respect to the β-actin signal and expressed as relative expression level. Values with dissimilar letters are significantly different ($p < 0.05$) from each other. Values are expressed as mean ± SD ($n = 5$).

Zhang et al. (1997) reported that TR mRNA is expressed in the human oocyte, and hence, TRs may have direct effects on the oocyte as well as on granulose and cumulus cells. Furthermore, Filby and Tyler (2007) reported a constant expression of TRα in the ovaries and testes and increased TRβ expression in the ovaries in fathead minnow, *P. promelas*. Considering these past results and the results of our present study, we suggest that TR genes are involved in the development and maturation of gonad and that TRβ plays a critical role in the cinnamon clownfish sex change to female. Although the involvement of TH in the reproduction of fish has been reported previously, information on the effects of the reproductive hormone on TH activity is limited (Roy et al. 2000).

We compared the expression of TR mRNA in gonad of immature cinnamon clownfish over time after GnRHa injection to examine the changes of TR genes. GnRH is the hormone that regulates the development of the gonad in vertebrates, including fish, and is involved in the sex change of gonads in fish (Soverchia et al. 2007). In the present study, two TRs increased after GnRHa injection. This indicates that GnRHa is involved in gonadal development by increasing the expression of TR mRNA in gonads of cinnamon clownfish. Although there have been limited studies

on the expression of TRs by GnRHa, we found that GnRHa regulated the pituitary–thyroid axis through T_4 in plasma, which was increased by GnRHa injection in sea lamprey, *Petromyzon marinus* (Sower et al. 1985) and carp, *Catla catla* (Roy et al. 2000). In addition, Roy et al. (2000) reported that GnRH directly stimulated the thyroid follicle and then synthesized TH in teleost fishes. One explanation for this is that GnRH, as a neurotransmitter, stimulates thyrotropin-releasing hormone (TRH) and then promotes thyroid-stimulating hormone (TSH) (Roy et al. 2000). Based on our results from this study, we suggest that GnRHa increased the synthesis and release of TH, which then increased the expression of TRα and TRβ mRNA.

In summary, we isolated full-length TRα and TRβ cDNAs in gonads of cinnamon clownfish and compared the expression of these genes during sex change from male to female. We found that TRs are involved in the maturation and development of gonads. To our knowledge this is the first study demonstrating changes in TR subtype expression as the gonads switch from testicular to ovarian tissue after removing the female fish, suggesting TR may be a potential modulator of this process. These findings support the hypothesis that TRs are involved in the control of male to female sex transition in cinnamon clownfish. Further studies are required to understand the role and activity of TH and TR in the maturation of teleost fishes.

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