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Tissue- and maturity-dependent expression pattern of androgen receptor mRNA in goldfish, *Carassius auratus*

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Androgen plays an important role in the regulation of gonadotropin production in vertebrates. We have investigated the transcriptional pattern of androgen receptor (AR) in a variety of tissues in maturing male and female goldfish by RT-PCR. Specific primer for AR was designed based on goldfish AR gene from the GenBank (accession number AY090897). AR was shown to be maturity- and tissue-dependent gene expression pattern in goldfish. In immature male goldfish, significantly higher transcript level of AR was observed in the pituitary and testis, compared to brain and liver. Mature male goldfish showed a similar expression pattern to immature male goldfish. Interestingly, when compare to male goldfish, female goldfish showed AR mRNA expression that was found to be weak in pituitary, and very low expression in brain. They could not be found to have expression in any other tissues. Taken together, the transcriptional analysis of AR depending on the tissue, sex, and maturity of a goldfish provides the opportunity for the study of goldfish reproductive physiology. The results provided for the first time a comparison of the tissue distribution of AR mRNA in sexually maturating male and female goldfish.

Keywords : Androgen receptor, Maturity, Goldfish, RT-PCR, Tissue distribution

Introduction

Steroid hormones produced by the gonads play crucial roles in sexual maturation and behavior in vertebrates (MacLuskey and Naftolin, 1981; Wilson *et al.*, 1981) including fish (Fostier *et al.*, 1981; Liley and Stacey, 1983; Kim *et al.*, 2002; He *et al.*, 2003). Androgens play an important role in male sexual differentiation by an androgen receptor (AR), which binds to specific DNA recognition sites and regulates transcription. The regulation of AR in target tissues has been investigated, and conflicting data regarding regulation of AR mRNA by androgen have been reported (Quarmby *et al.*, 1990; Blok *et al.*, 1992; Lin *et al.*, 1993; Shan *et al.*, 1995). These actions require binding of androgen to a specific receptor protein, AR. The resultant complex represents a trans-activation function that stimulates the transcription by binding to a specific DNA element termed a hormone-responsive element (Cato *et al.*, 1987).

AR gene has been cloned from several vertebrate species (Chang *et al.*, 1988; Lubahn *et al.*, 1988; He *et al.* 1990). AR is a member of a large superfamily of ligand-activated nuclear receptors, which also contain receptors for other steroid hormones (Brinkmann *et al.*, 1999; McKenna *et al.*, 1999). AR shares common features with other members of the ligand-activated nuclear receptors superfamily and their proteins can be divided into 4-5 dis-

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tinct domains (Krust *et al.*, 1986). AR contained the domains that characterize the steroid hormone receptor family, highly conserved C domain (DNA-binding domain; DBD), and moderately conserved E domain (ligand-binding domain; LBD) among species. It appears that the AR gene itself is subject to feedback regulation by androgens in the steroid hormone mechanism.

Seasonally breeding animals and/or avian species show a dramatic fluctuation in plasma androgen levels. Male canaries and other songbirds have high levels of circulating testosterone in the spring, when they establish territories and breed. After the breeding season, testosterone levels drop 10-fold and the testes regress (Nottebohm *et al.*, 1987). In the rainbow trout, *Oncorhynchus mykiss*, two isoforms of AR cDNA designated as ARα and ARβ have been reported (Takeo and Yamashita, 1999). Takeo and Yamashita (1999) demonstrated the rainbow trout ARα is predominantly expressed in the gonads whereas ARβ is expressed in low concentrations in pituitary and brain tissues. Similarly, another AR found recently in wrasse, *Halichoeres trimaculatus,* was highly expressed in the gonads and brain (Kim *et al.*, 2002). In the Atlantic croaker, *Micropogonias undulates,* two types of AR named as AR1 and AR2 have been identified and characterized (Sperry and Thomas 1999). The two receptors, AR1 and AR2, demonstrate widely different binding affinities for certain androgens, AR1 bound to be more specific for testosterone (Sperry and Thomas, 1999; Sperry and Thomas, 2000).

The information of the functional gene expression pattern of AR related to fish maturity and reproduction organs will provide the understanding of the steroid hormone mechanism for the sex maturity. In this study, to understand the mechanism of androgen action and AR regulation of gene transcription, the AR mRNA levels in various tissues from the goldfish were measured by RT-PCR. We also describe for the first time a comparison of the tissue distribution of AR mRNA in sexually maturing male and female goldfish.

Materials and methods

Experimental animals

Goldfish, *Carassius auratus*, ranged from 9 to 14 cm in length were purchased from Aquatic Imports (Calgary, Alberta), and kept at 17-18℃ in a semi-recirculating tank. The light regime was 16L/8D photoperiod. The fish were fed with commercial fish diet. Tissue samples from male and female fish at sexually immature and mature stages were removed and stored at -80℃ prior to use.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The goldfish AR cDNA sequence is available in GenBank under the accession number AY090897. The specific primers used to amplify AR sequence were designated as follows: AR primers: [5′-AAC TGC TCA GTG AGT GAA CG-3′] and [5′-ACT GGT GCA ACT TCC GAA CA-3′]. One μg of total RNA extracted from brain, pituitary, ovary, testis, and liver of goldfish were used for reverse transcription with an oligo(dT) primer and M-MLV reverse transcriptase (Gibco/BRL) according to the manufacture's instructions. Amplication of cDNA was carried out with 30 cycles; 94℃ for 45 sec for denaturing; 56℃ for 45 sec for primer annealing, and 72℃ for 1 min for extension, followed by 1 cycle of 5 min at 72℃ for extension. Goldfish 18S rRNA was used as an internal standard for normalizing mRNA levels. The goldfish 18S rRNR RT-PCR primers were designed as follows: 18S rRNA

primers: [5′-TCA GGG TTC GAT TCC GGA GA-3′] and [5′-CTC TAG CGG CGC AAT ACG AA-3′] from the GenBank (accession number AF047349).

Quantification of PCR amplified fragment was carried out by high resolution scanner and the band densities were estimated as described in a previous study (Choi and Habibi, 2003).

Statistical analysis

The results were presented as the mean ± the standard error (SE), and analyzed by a one-way ANOVA followed by the Duncan's multiple range tests were applied for statistical analysis.

Results

Structure and analysis of the goldfish AR gene sequence

We amplified goldfish AR (gfAR) gene from the published sequence (AY090897). Comparison of the amino acid sequence of gfAR with those of several species is shown in Fig. 1. The gfAR sequence was divided into four domains based on its sequence similarity to other steroid hormone receptors as defined by Krust *et al.* (1986). The gfAR amino acid sequence was found to have identity with rainbow trout, *O. mykiss*, ARα (59.0%) and β (57.4%) (Takeo and Yamashita 1999), black porgy, *Acanthopagrus schlegeli*, AR (52.6%) (He *et al.*, 2003) and red seabream, *Pagrus major*, AR (52.5%) (Touhata *et al.*, 1999). The domain, DBD, of the gfAR has the high level of identity (95.6-98.5%) when compared to the corresponding region of rainbow trout ARα, rainbow trout ARβ, red seabream AR, black porgy AR (Fig. 1). The domain, LBD, of the gfAR has 90.1% identity with the rainbow trout ARβ, 87.4% identity with the rainbow trout ARα, 85.8% identity with the red seabream AR, and 84.2% identity with the black porgy (Fig. 1). The other domains (A/B and D) show low homology.

Tissue distribution of the goldfish AR mRNA

We used the highly sensitive RT-PCR technique to survey the tissue distribution of gfAR mRNA in immature and mature male and female goldfish. The expression of 18S rRNA was monitored in all tissues, and then adjusted the amount of the gfAR mRNA to that of this housekeeping gene. In immature goldfish, AR was highly expressed in the pituitary, testis, and ovary while low transcript level of the gfAR was obtained in the brain (Fig. 2A, B).

In immature male goldfish, significantly higher transcript level of AR was observed in the pituitary and testis, compared to brain and liver (Fig. 2A). Mature male goldfish showed a similar expression pattern to immature male goldfish (Fig. 2B). When both immature and mature female goldfishes were compared to male goldfishes, AR transcription level was found to be weak in pituitary, and very low transcriptional level in brain (Fig. 3A, B). Which was not found to have expression in other tissues.

Discussion

Using the nomenclature of Krust *et al.* (1986), the gfAR amino acid sequence was consisted of four domains based on its sequence similarity to other steroid hormone receptors, A/B, C, D and E/F. The gfAR contains a high degree of conservation in the C (DNA-binding domain; DBD; 95.6%-98.6%) and E (Ligand-binding domain; LBD; 84.5-90.1%) domains when compare to rainbow trout ARs, red seabream AR, and black porgy AR

(Fig. 1). The other domains show low homology, which are 29.0-38.2% identity for A/B domain and 55.3-65.8% identity for D domain. In the A/B domain, however, a conserved region (residues 158-172 in the gfAR) is found among the goldfish, rainbow trout, red seabream, and black porgy ARs. It has been shown that the A/B domain contains transactivation function, which is dependent on the cell and the promoter types but less on the ligand-binding (Truss and Beato, 1993, Todo *et al.*, 1999). Thus, it is interesting to determine whether the conserved regions in the A/B domains of ARs is involved in common functions among ARs, such as cell/promoter-specific transactivation function. In the DBD, the position of cysteine residues, which constitute the two zinc finger motifs and the P box (GSCKV), is the important region for determination of the target gene (Umezono and Evans, 1989) and is also conserved in the gfAR. In addition, a leucine zipper motif is found in the LBD

(A)

C domain (DNA-binding domain; DBD)

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Goldfish  481:RTCLICSDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTIDKLRRKNCPSCRL 540
R.troutA  496:RTCLICAEEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTIDKLRRKNCPSCRL 555
R.troutB  495:RTCLICADEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTIDKLRRKNCPSCRL 554
Seabream  411:RMCLICSDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTIDKLRRKNCPSCRL 470
BL.porgy  413:RMCMICSDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASKNDCTIDKLRRKNCPSCRL 469

Goldfish  541:RKCFEAGM                                                     548
R.troutA  556:RRCFEAGM                                                     563
R.troutB  555:RKCFEAGM                                                     562
Seabream  471:RKCFEAGM                                                     478
BL.porgy  470:RKCFEAGM                                                     480
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E/F domain (Ligand-binding domain; LBD)

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Goldfish  587:TFHSQLIFLNILESIEPEVVNAGHDHAQPDSAVALLTSLNELGERQLVKVVKWAKGLPGF 646
R.troutA  602:SFHSQLVFLNILESIEPEVVHAGHDQCQPDSAAALLTSLNELGERQLVKVVKWAKGLPGF 661
R.troutB  601:TFHSQLVFLNILESIEPEVVNAGHDHCQPDSAAVLLTSLNELGERQLVKVVKWAKGLPGF 660
Seabream  517:SFNSQVVFLNVLESTEPEVVNAGHDYGQPDSAATLLTSLNELGERQLVKVVKWAKGMPGF 576
BL.porgy  519:SFNSQVVFLNILESIEPEVAYAGHDYGQPDSAATLLTSLNELGEKQLVKVVKWAKGLPGF 578

Goldfish  647:RNLHVDDQMTVIQHTWMGVMVFALGWRSYKNANARMLYFAPDLVFNDHRMHISSMYEHCV 706
R.troutA  662:RNLHVDDQMTVIQHSWMGVMVFGLGWRSYKNANCRLLYFAPDLVFNDHRMHISSMFDHCI 721
R.troutB  661:RNLHVDDQMTVIQHSWMGVMVFGLGWRSYKNVNARMLYFAPDLVFNDHRMHISSMFEHCI 720
Seabream  577:RNLHVDDQMTVIQHSWMGVMVFGLGWRSYKNVNGRMLYFAPDLVFNEHRMHISTMYEHCI 636
BL.porgy  579:RNLHVDDQMTVIQYSWMGVMVFGLGWRSYKNVNGRMLYFAPDLVFNEHRMHISSMYEHCI 638

Goldfish  707:QMKHLSQEFVLLQVTQEEFLCMKALLLFSIIPVEGLKSQKYFDDLRLTYINELDRLINYG 766
R.troutA  722:RMRQLSQEFVLLQVTQEEFLCMKALLLFSIIPVDGLKSQKYFDELRLTYINELGRVINYG 781
R.troutB  721:RMRHLSQQFVLLQVTQEEFLCMKALLLFSIIPVDGLKSQKYFDELRLTYINELDRVINYG 780
Seabream  637:RMRHLSQEFLLLQITQEEFLCMKALLLFSIIPVEGLKSQKYFDELRLTYINELDRLINYR 696
BL.porgy  639:RMRHLSQEFLLLQITQEEFLCMKALLLFSIIPVEGLKSQKYFDELRLTYINELDRLISYR 698

Goldfish  767:RKTNCAMRFQQLTRLMDSLQPIVRKLHQFTFDLFVQARSLPTKVSFPEMIAEIISVQVPK 826
R.troutA  782:RKSNQSQRLYQLTRLMDSLQPVVRKLQQFTFDLFVQAQSLPTKVNFPEMIAEIISVHLPK 841
R.troutB  781:RKSNQSQRFYQLTRLMDSLQPIVRKLQQFTFDLFIQAQSLPTKVSFPEMIAEIISVHVPK 840
Seabream  697:MNTNCSQRFYQLTRLLDSLQMTVKKLHQFTFDLFVQAQSLPTKVSFPEMIGEIISVHVPK 756
BL.porgy  699:MSANCSQRFYQLTRLLDSLQMTVKKLHQFTFDLFVQAQSLPTKVSFPEMIGEIISVHVPK 758

Goldfish  827:ILAGLSKPILFHK                                                839
R.troutA  842:ILAGLAKPILFHK                                                854
R.troutB  841:ILAGLAKPILFHK                                                853
Seabream  757:IPAGLAKPILFHE                                                769
BL.porgy  759:ILAGLAKPILFHD
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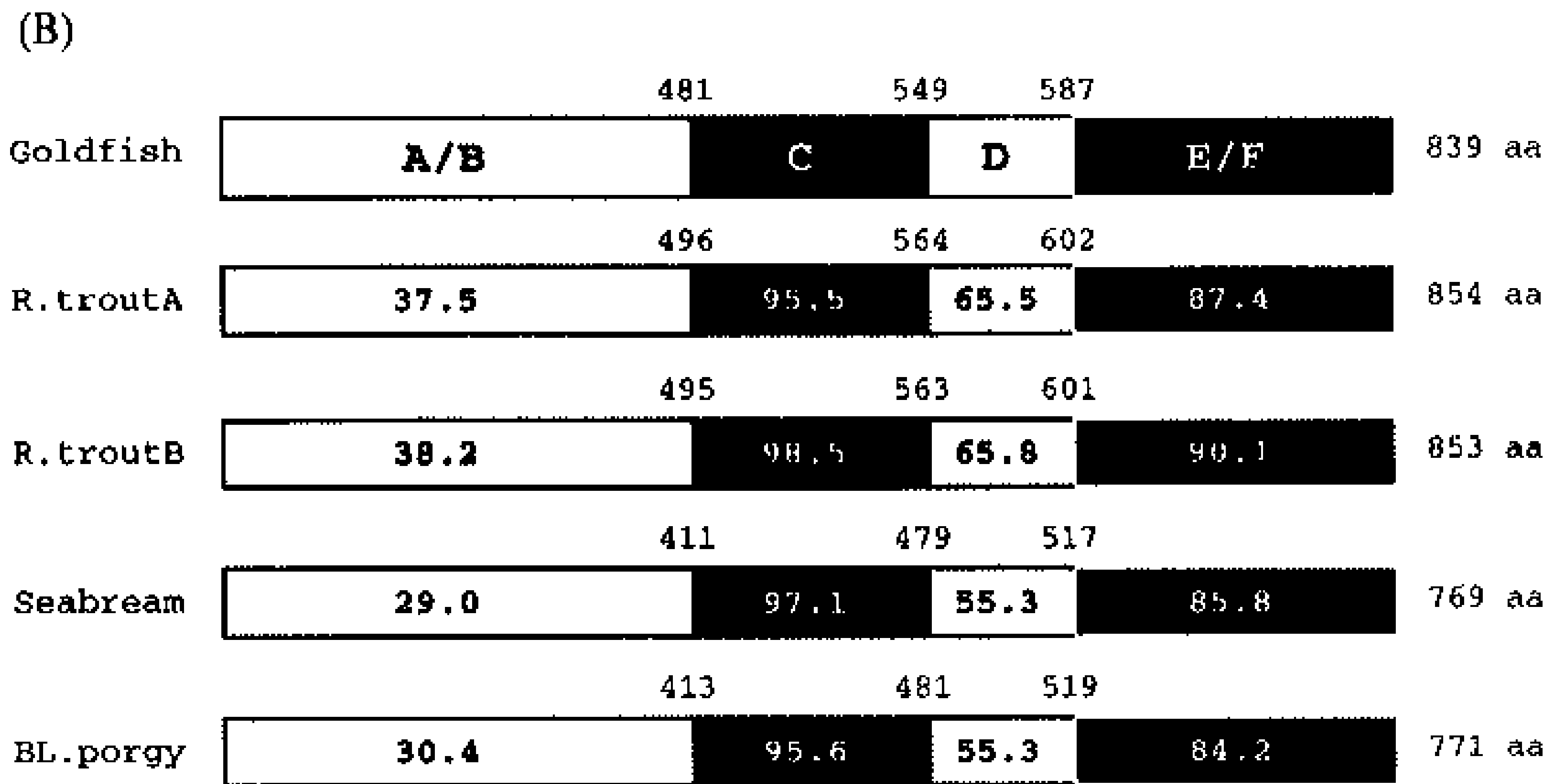

Fig. 1. (A) Comparison of amino acid sequences of the C (DNA-binding domain; DBD) and E domains (Ligand-binding domain; LBD) of the goldfish androgen receptor (AR), rainbow trout ARα (R.troutA), rainbow trout ARβ (R.troutB), red Seabream AR (Seabream) and black porgy AR (BL.porgy). Amino acid sequences were optimally aligned to match identical residues, which are indicated by shaded boxed. The sequences were brought from the Genbank/EMBL/DDBJ sequence databases. AR sequences used for alignment are goldfish AR (this paper, AY090897), rainbow trout ARα (AB012095), rainbow trout ARβ (AB012096), red seabream AR (AB017158) and black porgy (AY219702). (B) Domain structure of the goldfish AR and identity with rainbow trout ARs, red seabream AR and black porgy AR. The percentage of amino acid identity of each domain relative to the goldfish AR is indicated within the box representing the corresponding domain.

region of the gfAR (residues 777-806). The leucine zipper-like structure is conserved in many nuclear receptors and is required for dimerization of receptor (Pfahl, 1993).

Our results revealed an interesting tissue-specific difference in the regulation of AR mRNA. In this study, we also compared the transcriptional pattern of gfAR in a variety of tissues in related to goldfish sex and maturity by RT-PCR. The transcription level of AR was strongly expressed in male goldfish pituitary and testis (Fig. 2A, B). Also there was low expression in brain and liver tissues from male goldfish (Fig. 2A, B). In immature male goldfish, AR expression showed a similar pattern as mature male goldfish. Surprisingly, however, when compare to male goldfish, female goldfish showed AR mRNA expression that was found to be weak in pituitary, and very low expression in brain. They could not be detected in any other tissues (Fig. 3A, B).

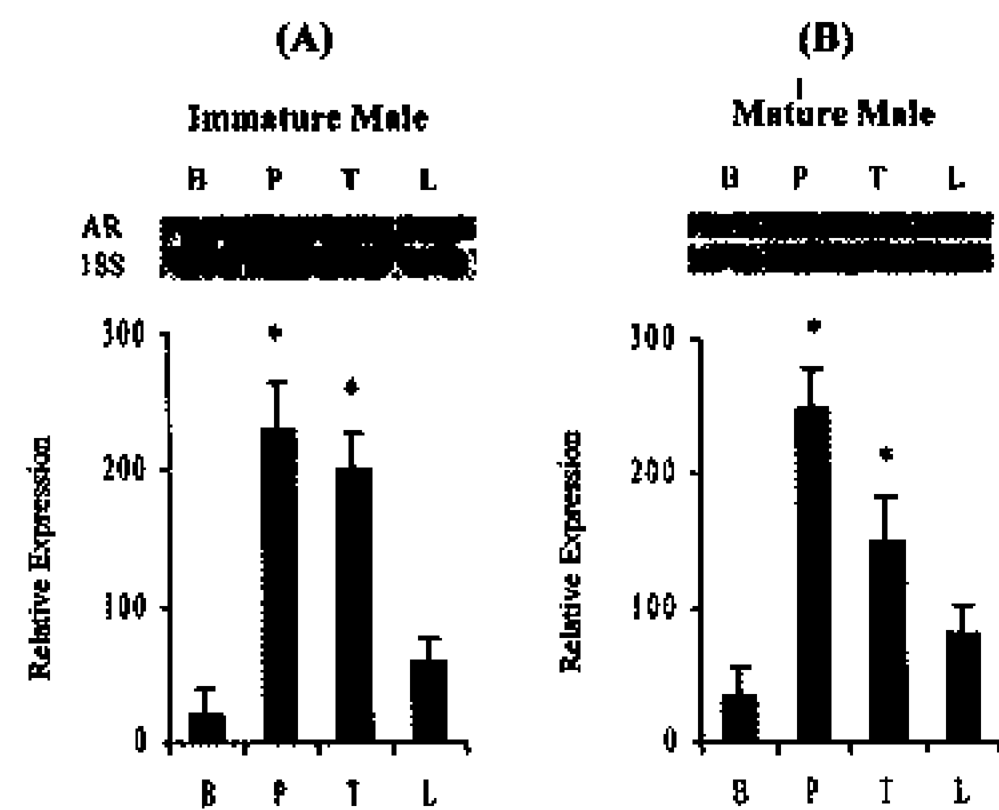

Fig. 2. One microgram of total RNA prepared from brain (B), pituitary (P), testis (T) and liver (L) was used for reverse transcription and amplified in immature and mature male goldfish using AR specific primer. Tissue distribution of goldfish AR was analyzed by RT-PCR. The expression of goldfish 18S rRNA mRNA was evaluated in each RT reaction product as a control. The expression level of each tissue was normalized with respect to the goldfish 18S rRNA signal, and expressed as relative expression level. Values with dissimilar letters are significant different ($P<0.05$) from each other. Values are mean ± the standard error (SE) of these five experiments, each using separate male goldfish.

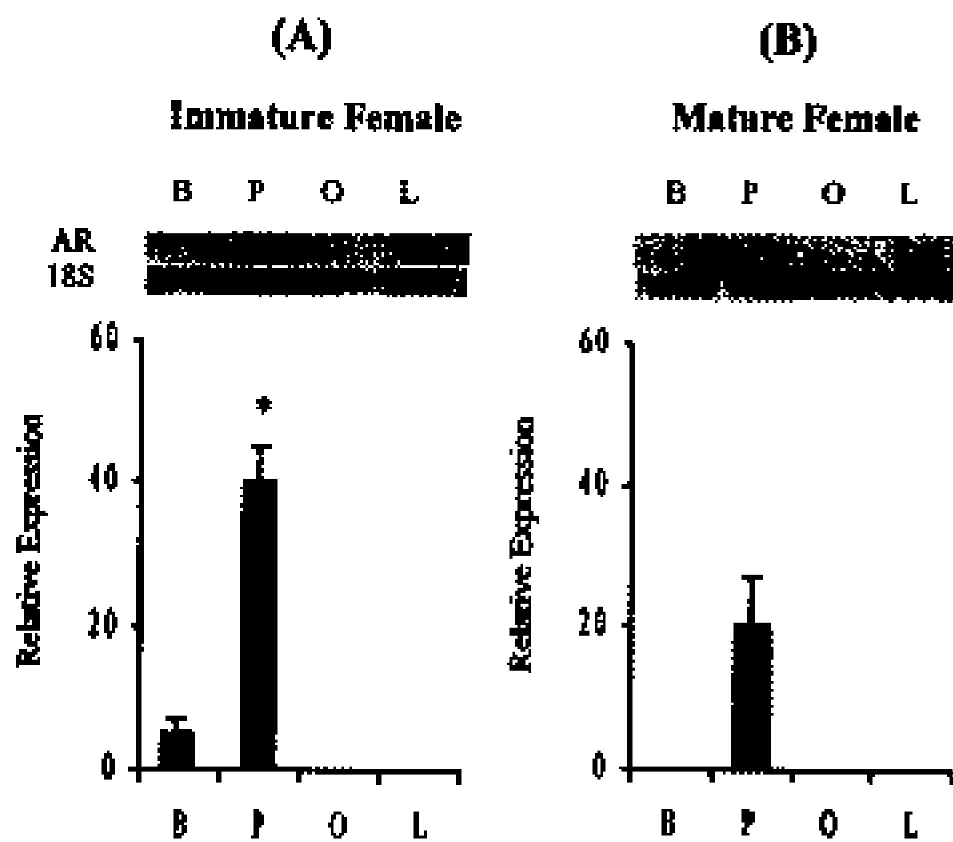

Fig. 3. Details as described in Fig. 2 using ovary (O) and other tissues from immature and mature female goldfish.

The findings suggested that gfAR may play important roles in the regulation of pituitary, and ovarian/testicular function during sexually maturing in male goldfish. At present, little information is available on the relative importance of AR in the regulation of growth and reproduction in goldfish and other teleosts species.

In recent discovery, it has been published that precedence for isoforms of two types of AR was set in rainbow trout (Takeo and Yamashita, 1999). For rainbow trout ARα, strong signals were found in the ovary and testis, followed by weaker signals in brain, pituitary, and liver (Takeo and Yamashita, 1999). Weak expression of ARβ was detected in the pituitary, and very low expression in testis and brain (Takeo and Yamashita, 1999).

These present results are in accordance with findings in rainbow trout's concerning tissue specific expression of AR. Interestingly, the expression pattern of the gfAR in immature goldfish is similar to expression of rainbow trout ARα. RT-PCR analysis revealed that the gfAR expression in the mature goldfish showed a similar expression pattern of rainbow trout ARβ. Moreover, the deduced amino acid sequence of the gfAR has a high similarity with rainbow trout ARβ (78.4% similarity), which is better than rainbow trout ARα. Therefore, it is possible that goldfish has two AR subtypes, which one is expressed in immature and the other is involved in mature goldfish tissues. Thus, further work using different approach such as a screening of cDNA library is required to determine whether the goldfish has the multiple AR subtypes as reported in rainbow trout.

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