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Thyroid hormone receptor β is widely expressed in the brain and pituitary of the Japanese eel, *Anguilla japonica*

Y. Kawakami [a,*], S. Adachi [b], K. Yamauchi [b], H. Ohta [a]

[a] *Department of Fisheries, Graduate School of Agriculture, Kinki University, Nara 631-8505, Japan*
[b] *Division of Marine Biosciences, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan*

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Abstract

In a previous study, we cloned the thyroid hormone receptor βB (TRβB) from a teleostean fish, the Japanese conger eel (*Conger myriaster*). The gene encoding this receptor is expressed in the brain and pituitary. In this study, we cloned TRβB from the brain of the Japanese eel (*Anguilla japonica*) to extend our studies into thyroid hormone function in fish. RT-PCR analysis demonstrated that the TRβB transcripts were abundant in both the brain and pituitary. The TRβB cDNA encoded a 379 amino acid protein with much higher homology to the conger eel TRβB than to other fish TRs, supporting the existence of a TRβB isoform, at least in anguilliforms. In a transiently transfected Japanese eel cell line, Hepa-E1, TRβB and its splice variants in the ligand-binding domain (TRβBL) showed thyroid hormone (TH)-dependent activation of transcription from the TH-responsive promoter. *In situ* hybridization studies revealed the presence of TRβB transcripts in the pars distalis of the pituitary.

Keywords: Thyroid hormone receptor β; Thyroid-stimulating hormone cells; Pituitary; Japanese eel

1. Introduction

Thyroid hormones (THs) are involved in growth, differentiation, metamorphosis and reproduction, and their pleiotropic effects are mediated through thyroid hormone receptors (TRs). TRs comprise part of a superfamily of nuclear receptors that are characterized by distinct regions or domains for DNA binding and ligand-binding, respectively, separated by "hinge" regions that may target the receptor to the nucleus (Evans, 1988; Lazar and Chin, 1990). In mammals, the TRα and β subtypes are the products of two distinct genes, whereas the two TRβ isoforms (TRβ1and TRβ2) are splice variants from the same gene. In mammals, TRβ1 is widely expressed and is particularly abundant in the liver and kidney (Lazar, 1993). Conversely, in mammals TRβ2 is most highly expressed in the pituitary (Hodin et al., 1989; Cook et al., 1992), but mRNA and immunoreactive material have also been detected in the hypothalamus and the developing ear (Bradley et al., 1992; Cook et al., 1992; Lechan et al., 1993) as well as in adult bodily tissues albeit at low mRNA levels (Schwartz et al., 1994).

We have previously reported the existence of TRs in a teleostean fish, the Japanese conger eel (*Conger myriaster*, *Anguilliformes*: *Congridae*). In this species, two TRαs and two TRβs were identified, implying that in teleostean fish there are at least four TR isoforms (Kawakami et al., 2003a,b). Japanese conger eel TRβ2 is specifically expressed in the brain and pituitary, presenting a TR expression pattern that is, so far, unique amongst teleostean fish. Indeed, the expression pattern much more resembles that of TRβ2 in mammals (Kawakami et al., 2003a), and to avoid confusion between subtypes, we therefore propose the re-definition of TRβ1 and TRβ2 in anguilliformes as TRβA and TRβB, respectively. It is noteworthy that unlike in mammals, in which TRα and TRβ are the products of different

* Corresponding author. Fax: +81 743 75 5966.
E-mail address: yutakakjp@yahoo.co.jp (Y. Kawakami).

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genes and TRβ has splice variants, the distinct TRβ isoforms in the conger eel (cTRβA and cTRβB) are concluded to be produced by different genes, and each of these isoforms (cTRβA and cTRβB) themselves have splice variants.

Anguilliforms undergo a typical metamorphosis in their development from leptocephalus to elver (Smith, 1979) and it is well known that in the Japanese conger eel, metamorphosis occurs following the addition of THs (Kitajima et al., 1967; Yamano et al., 1991). During the metamorphic stage of the Japanese conger eel, the expression pattern of cTRβA mRNA was highest during metamorphic climax and high levels of expression were maintained after metamorphosis. It was also shown that cTRβB is highly expressed in the brain and pituitary gland of larvae undergoing metamorphosis, and that cTRβB mRNA peaked in the elver after metamorphosis. Thus, we proposed that TRβB plays an important role in the regulation of the hypothalamic-pituitary-thyroid axis in the conger eel (Kawakami et al., 2003b). However, a comparable expression pattern of TR, similar to that of cTRβB, has not been reported in other teleostean fish, and generalizations may not hold across taxa. It is therefore necessary to establish the existence of TR subtypes, including the likes of cTRβB, and to determine the role of TRs in different fish species. Thyroid-stimulating hormone (TSH), which consists of an α-subunit and a TSH-specific β-subunit, is most important in the hypothalamus-pituitary-thyroid axis. It is clear that TR, especially TRβ, plays a critical role in the regulation of TSH (Refetoff et al., 1993). Furthermore, it is important to identify whether TRβB is expressed in TSH-producing cells in the fish pituitary.

The Japanese eel (*Anguilla japonica*, *Anguilliformes*: *Anguillidae*) is a typical anguilliform, with a morphology during the metamorphic stage very similar to that of the Japanese conger eel. It has the potential to become an important model fish for research into TH function, because offspring of the Japanese eel have now been successfully produced, which is the first time this has been achieved for anguilliforms (Tanaka et al., 2001). The purpose of this study is to investigate TH function in anguilliforms. At first, we undertook the cloning and characterization of the cDNA encoding TRβB in the Japanese eel and we then analyzed the expression pattern of TRβB in a variety of adult organs, especially the pituitary, using RT-PCR and histological analysis, especially focusing on comparison with cTRβs.

2. Materials and methods

2.1. Animals

Cultivated adult male Japanese eels (150–200 g body mass) were purchased from a commercial eel supplier. Tissues of adult Japanese eel were frozen in liquid nitrogen and kept at −80 °C prior to the preparation of total RNA or Poly(A) + RNA. Eel brain with pituitary tissue was used for the construction of cDNA for rapid amplification of cDNA ends by (RACE)-polymerase chain reaction (PCR) (Kawakami et al., 2003a).

2.2. Reverse transcription (RT)-PCR and cDNA cloning

Total RNA was extracted from liver using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Poly(A) + RNA was subsequently isolated from total RNA with Oligotex-dT-30 (Takara, Otsu, Japan). Isolated RNA was denatured at 70 °C for 10 min, placed on ice, reverse transcribed with Superscript II (Invitrogen) at 42 °C for 40 min and 50 °C for 30 min, using oligo$(dT)_{12\text{-}18}$ and stopped at 70 °C for 10 min.

For amplification of Japanese eel TRβB (eTRβB) cDNA fragments, sense and antisense degenerate primers were designed as described in Kawakami et al. (2003a). PCR was carried out in a final volume of 50 μL containing 0.5–1 pg cDNA, 400 nM of each primer (Table 1), 800 μM of each dNTP and 2.5 U Ex Taq (Takara). PCR was carried out for 35 cycles using a Thermal Cycler Dice Gradient (Takara) under the following conditions: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Following PCR, products were checked using 1% agarose gel electrophoresis and selected bands were cut out and isolated using a QIAprep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands). Amplicons were subcloned into the plasmid vector pGEM-T Easy (Promega, Madison, WI, USA), using a Ligation-Convenience Kit (Nippon Gene, Tokyo, Japan). After subcloning, positive clones were sequenced with dye terminator cycle sequencing (DTCS) Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) using a CEQ™ 8800 (Beckman Coulter).

Table 1
Primers used for cloning, PCR and *in situ* hybridization analysis of Japanese eel thyroid hormone receptor βBs (eTRβBs)

Name	Primer sequence	Nucleotide number of annealing site (bp) (Fig. 1)
NP 1	5′-TGGGCGTGTCCCTGTCCGCCTTCAACC -3′	958–984
NP 2	5′-GCTCCTGCAAGCCGTCATCCTCCTGAG-3′	1004–1030
NP 3	5′-ACCTTCATCAGGAGCTTGGGCCAG-3′	1142–1165
NP 4	5′-TCCTCCTGGCAGCGCTCGATCCGC-3′	1061–1084
eTRβB-UTR-S	5′-AGAGCACGCCGGAACACTGAAG-3′	1–22
eTRβB-UTR-A	5′-AATTCAACATTACAAAGTCAGCTAGC-3′	1797–1822
eTRβB-T7	5′-TAATACGACTCACTATAGGGACCAAGTCCGTCGCCATGCC-3′	
eTRβB-SP6	5′-GATTTAGGTGACACTATAGAAGAGCACGCCGGAACACTGA-3′	
eTRβB-S	5′-TGGAGGATATGAGGATGAAGACT-3′	38–60
eTRβB-A	5′-GTTTCTGCTTCCAGTGGTTGC-3′	511–531
*Hin*dIII-eTRβB-S	5′-AAGCTTTGAGCCCTGAGGTGTGGCA-3′	
*Bam*HI- eTRβB-A	5′-GGATCCAGACCGTCAGTCCTCGAAC-3′	
β-Actin-S	5′-GGAGAAGAGCTACGAGCTGCC-3′	
β-Actin-A	5′-CAATGATCTTGATTTTCATTGTGCT-3′	

Primers for amplification of eTRβB cDNA fragment; annealing primers with NPs (nested primers), gene-specific primers for 3′- and 5′-RACE-PCR of eTRβBs; eTRβB-UTRs (untranslated regions), sense and antisense primers for sequencing of eTRβBs containing an open reading frame.