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Thyroid hormone receptor expression during metamorphosis of Atlantic halibut (*Hippoglossus hippoglossus*)

Malyka Galay-Burgos [a], Deborah M. Power [b], Lynda Llewellyn [a], Glen E. Sweeney [a,*]

[a] *School of Biosciences, University of Wales, Museum Avenue, CF10 3US Cardiff, UK*
[b] *Comparative Molecular Endocrinology Group, Marine Science Centre (CCMAR), Universidade do Algarve, 8005-139 Faro, Portugal*

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Abstract

Flatfish such as the Atlantic halibut (*Hippoglossus hippoglossus*) undergo a dramatic metamorphosis that transforms the pelagic, symmetric larva into a benthic, cranially asymmetric juvenile. In common with amphibian metamorphosis, flatfish metamorphosis is under endocrine control with thyroid hormones being particularly important. In this report we confirm that tri-iodothyronine (T_3) levels peak at metamorphic climax during halibut metamorphosis. Moreover, we have isolated cDNA clones of TRα and TRβ genes and confirmed the presence in halibut of two TRα isoforms (representing the products of distinct genes) and two TRβ isoforms (generated from a single gene by alternative splicing). Real-time PCR was used to assess expression of these genes during metamorphosis. TRβ shows the most dramatic expression profile, with a peak occurring during metamorphic climax.

Keywords: Atlantic halibut; Flatfish; Metamorphosis; Thyroid hormones; Thyroid hormone receptor

1. Introduction

The link between thyroid hormones (TH) and the onset and progression of amphibian metamorphosis (reviewed by Brown and Cai, 2007; Tata, 2006; Sachs et al., 2000) has been known for almost a century (Gudernatsch, 1912). More recent research, notably some incisive and elegant transgenic animal studies, has emphasised the importance of thyroid hormone receptors (Buchholz et al., 2006, 2004; Schreiber et al., 2001) and iodothyronine deiodinases in the process (Brown, 2005; Cai and Brown, 2004). However, amphibia are not the only vertebrates that undergo metamorphosis. The development of fish involves a metamorphosis from larval to juvenile form that is manifest most dramatically in flatfish (pleuronectiformes) such as sole, halibut and flounder. In these species, the pelagic larva undergoes extensive remodelling which leads to a loss of external bilateral symmetry and generates a benthic juvenile with both eyes on the same, pigmented, side of the body. These morphological changes are scarcely less profound than those of amphibian metamorphosis but are clearly distinct from them. On the other hand, some of the biochemical and physiological changes that accompany metamorphosis, such as switching of globin (Wakahara and Yamaguchi, 2001; Miwa and Inui, 1991) and keratin (Campinho et al., 2007a) types and the production of new isoforms of muscle proteins (Yamano et al., 1991a; Campinho et al., 2007b) are similar in flatfish and amphibia. Still more significantly, there is strong evidence that thyroid hormones regulate metamorphosis in flatfish in much the same manner as in amphibia. Hence, metamorphosis in Japanese flounder (*Paralichthys olivaceus*) and Atlantic halibut (*Hippoglossus hippoglossus*) is induced precociously by TH treatment and Japanese flounder metamorphosis is delayed or abolished following exposure to agents that inhibit TH synthesis (De Jesus et al., 1993; Solbakken et al., 1999).

THs exert their genomic effects by binding to thyroid hormone receptors (TRs). THs also have non-genomic effects (Davis and Davis, 1996), some of which are also mediated via the TRs mentioned above (Hiroi et al., 2006). However, the genomic actions alone of thyroid hormones may be sufficient for metamorphosis in amphibia (Buchholz et al., 2004). TRs are members of the nuclear receptor superfamily, and act as hormone-regulated transcription factors (reviewed in Zhang and Lazar, 2000; Oetting and Yen, 2007). They usually activate the

* Corresponding author. Tel.: +44 2920874123; fax: +44 2920874116.
E-mail address: sweeneyge@cf.ac.uk (G.E. Sweeney).

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transcription of target genes when complexed with hormone but act as repressors in the absence of ligand, although some genes are repressed rather than induced by THs. There are two principal isoforms of TR in vertebrates, designated TRα and TRβ, which are the products of distinct genes. The mammalian TRα and TRβ genes produce a variety of additional receptor isoforms via alternative splicing and the use of alternative transcription start sites (Lazar, 1993; Flamant and Samurat, 2003). The functions of TRα and TRβ have been extensively investigated in transgenic mice and it is clear that, *in vivo*, they have both common and isoform-specific roles (reviewed by Cheng, 2005; Flamant and Samurat, 2003). For example, TRβ isoforms are more important than those of TRα in the inner ear (Forrest et al., 1996a), liver, and in TH homeostasis (Forrest et al., 1996b), whereas TRα isoforms play a more significant role in the heart (Mai et al., 2004) and GI tract and also in temperature regulation. These differences in function *in vivo* between TRα and TRβ may arise as a result of slight biochemical differences between the isoforms (for example, see Guissouma et al., 2002). However, microarray analysis of TH-induced gene expression in liver from TRα and TRβ deficient mice indicates that the two isoforms regulate almost the same set of genes (Yen et al., 2003). Hence, some or all of the functional differences between the receptor types may simply be a reflection of differences in their expression profiles.

The teleosts also possess TRα and TRβ but, in contrast to mammals, Japanese flounder, conger eel (*Conger myriaster*) and Atlantic salmon (*Salmo salar*) have two distinct TRα genes (Yamano et al., 1994; Marchand et al., 2001; Jones et al., 2002; Kawakami et al., 2003a). Conger eel also has two TRβ genes, and whilst only a single TRβ gene has been found in other teleosts examined to date, further TRβ isoforms have been described that result from alternative splicing (Yamano and Inui, 1995; Marchand et al., 2001; Kawakami et al., 2003b). We have previously described (Llewellyn et al., 1999) a full-length TRα (henceforth designated as TRαA) from a commercially important flatfish species, the Atlantic halibut (*H. hippoglossus*). Here we describe partial clones of a second TRα gene (designated TRαB) and the TRβ gene from this key species. Real-time PCR was subsequently used to assess the expression of all three TR genes during metamorphosis and has been related to changes in whole body TH concentration.

2. Materials and methods

2.1. Sampling

Halibut larvae were cultured using standard commercial procedures. Broodstock fish were stripped for eggs and milt, and eggs were fertilised *in vitro* and then placed in open system egg incubators at 5.0–5.5 °C for 14 days and then transferred to open system silos. For the next 45–50 days the larvae were kept in the dark at a temperature of 5–6 °C, after which they were transferred to the first feeding tanks at 11 °C and fed live feed (*Artemia*) for approximately 60 days. After metamorphosis the bottom dwelling juveniles were transferred to weaning tanks and gradually weaned for 10 days on dry feeds. Samples were taken from a standard commercial production cycle (Fiskeldi Eyjafjarðar Ltd., Iceland) at regular intervals and larvae were staged according to the criteria of Sæle et al. (2004). Larvae were stored at −20 °C in RNAlater prior to RNA extraction using Tri-reagent.

2.2. TH extraction and radioimmunoassay

The T_4 and T_3 content of halibut larvae was assessed by specific radioimmunoassay (RIA). Individual larvae of stage 5 ($n = 10$), stage 6 ($n = 10$), stage 7 ($n = 8$), stage 8 ($n = 7$), stage 9 ($n = 6$) and stage 10 ($n = 6$) were extracted in methanol, re-extracted in 50 μl methanol, 200 μl chloroform and 100 μl barbital buffer, and centrifuged (3000 rpm for 30 min at 4 °C). Then, the upper phase was removed, lyophilised, reconstituted in assay buffer, heat treated (65 °C, 2 h) and assayed. Assays for both T_3 and T_4 were highly specific and reproducible and were performed under equilibrium conditions using anti-T_3 and anti-T_4 sera (Sigma–Aldrich) as previously described (Einarsdottir et al., 2006). Statistically significant differences in the concentration of T_4 or T_3 detected for different stages were compared by ANOVA on log transformed data, followed by All Pairwise Multiple Comparison Procedures (Holm–Sidak method). Results were considered to be significantly different at $p < 0.05$.

2.3. Cloning of TRs

PCR using degenerate primers was used in order to isolate partial clones of halibut TRs. A multispecies alignment was performed using TR sequences from a variety of vertebrate species and degenerate PCR primers were designed that corresponded to particularly well conserved motifs. One primer pair (forward primer: AATGYCGCTTCAARAARTGYAT, reverse primer: GTAAACTGRCTRAAGGCYTC) was used in RT-PCR reactions in which the substrate was RNA isolated from metamorphosing halibut larvae. The PCR products generated were gel purified, subcloned into pGEM-T (Promega, UK) and sequenced.

2.4. Sequence analysis

Complete or partial nucleotide sequences of TRs from *Fugu rubripes* were obtained by BlastN searching of the *Fugu* genome (http://fugu.biology.qmul.ac.uk) with the halibut TRαA, TRαB and TRβ sequences. The intron–exon structure of the *Fugu* TRβ gene was deduced using Spidey (http://www.ncbi.nlm.nih.gov/spidey/) to identify sequences within the individual *Fugu* scaffold M001021 that showed strong similarity with known teleost TR mRNAs, followed by manual comparison of the putative splice sites in the *Fugu* sequence with the consensus sequences for vertebrate 5′ and 3′ splice junctions.

2.5. Real-time RT-PCR analysis of gene expression

RNA was prepared from five replicate individuals from stage 5 (larvae prior to onset on metamorphosis) through to stage 10 (fully metamorphosed juveniles) using Tri-reagent (Sigma–Aldrich) and subsequently treated with DNAse (Ambion) according to the manufacturer's instructions. cDNA was prepared from 0.5 μg aliquots of each RNA using random hexamer primers and superscript II reverse transcriptase (Invitrogen).

Expression of the genes encoding TRα1, TRα2 and TRβ was quantified using real-time RT-PCR using fluorogenic 5′ nuclease assays performed on an ABI PRISM® 7700 Sequence Detection System (Perkin-Elmer Biosystems, UK). The sequences of the primers and TaqMan probes used in each assay were as follows: TRαA, primers TTGTTGGACATTGGCTCCATT and CCGCCTCATTGTCCTGTGAT, probe TGCCTCAGTACCGTCCAGCGG; TRαB, primers TGCGTCGCTCCCTGTTCT and TTGGTGCTGGACGACTCAAA, probe TCGATCAGACGCCGCTTCGCT; TRβ, primers TGGTGACTGACGCCCATATG and CAGGAATTTCCGCTTCTGCTT, probe CCACGAATGCCCAGGGCAACC. RT-PCR assays, using 5% of the cDNA generated in the reactions described above, were normalised with respect to expression of 18s rRNA, for which the primers were GCATGCCGGAGTCTCGTT and TGCATGGCCGTTCTTAGTTG, and the probe sequence was CCACGAATGCCCAGGGCAACC. Isoform specificity of the probes and primers was confirmed by experiments in which each primer/probe combination was tested for its ability to amplify DNA from the plasmid clones of TRαA, TRαB and TRβ. Each individual sample was analysed in triplicate. One-way analysis of variance (ANOVA) was used to assess differences between means, after testing for homogeneity of group variances and normality of residuals. Tukey post hoc tests subsequently identified which stages were significantly different ($p < 0.05$) from the others for each of the genes analysed.