Cloning and expression of a thyroid hormone receptor α1 in the perennibranchiate amphibian Proteus anguinus

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ABSTRACT We demonstrated the presence of thyroid hormone receptor α mRNAs in tissues of the perennibranchiate amphibian Proteus anguinus, which is insensitive to thyroid hormone. From P. anguinus muscle we cloned and sequenced the 3' coding and untranslated region of a cDNA corresponding to a thyroid hormone receptor α1. Using cDNA-PCR and in situ hybridization, we showed a tissue-specific expression of thyroid hormone receptor α genes, which was not upregulated by thyroid hormone as opposed to that observed in the TH-sensitive species, Xenopus laevis.

KEY WORDS: thyroid hormone receptors, perennibranch amphibian, Proteus anguinus, RT-PCR, in situ hybridization

Introduction

Amphibian metamorphosis is a complex biological event which totally depends on the presence of thyroid hormone (Gilbert and Frieden, 1981). Thyroid hormone – T3 being the physiologically most potent form – enters the cell and proceeds to the nucleus where it binds to thyroid hormone receptors (THR) (Glass and Holloway, 1990). Ligand-bound THR complexes modulate the transcription of thyroid hormone-responsive genes (Glass et al., 1987; Forman et al., 1988; Darling et al., 1989). In mammals, chicken and anuran amphibians, the members of the two known THR families, α and β have been cloned and characterized (reviewed in Glass and Holloway, 1990; Yaoita et al., 1990; Kanamori and Brown, 1992; Shi et al., 1992). In Xenopus, the mRNA for THRα increases just before tadpoles become competent to respond to exogenous TH while the THRβ mRNA level increases in synchrony with the endogenous TH concentration during metamorphosis, suggesting distinct functions for THRα and THRβ (Yaoita and Brown, 1990).

Among salamanders, the perennibranchiate species is described as "obligate neotenes" since they never undergo metamorphosis and are insensitive to massive doses of thyroid hormone. However, they possess thyroid glands, the products of which can induce metamorphosis in other species (Lynn, 1961).

In amphibians, muscle development is characterized by a larval to fast myosin heavy chain transition regulated by thyroid hormone during anatomical metamorphosis (Chanoine et al., 1987). In the hypothyroidic A. mexicanum, where metamorphosis does not occur spontaneously, this transition is partial; larval and fast isoforms are coexpressed in adult muscles. Experimental hyperthyroidism involves anatomical metamorphosis and a complete transition from larval to fast isoforms in this species. In the perennibranchiate P. anguinus, there is also a partial myosin isoform transition but long term T3 treatment fails to induce a complete transition (Chanoine et al., 1989). In connection to this, Dent (1968) noted that P. anguinus skin does not respond to thyroxin(T4). The presence of thyroid hormone receptors in perennibranchiate tissues has been analyzed but the results were contradictory. Galton and Germain (1985) suggested the presence of a low THR protein number in red blood cells of Necturus maculosus while more recently, Yaoita and Brown (1990) suggested that the low receptiveness of the perennibranchiate tissues to the thyroid hormone could be due to a lack of THR mRNAs.

In a previous short report (Ho Huynh et al., 1993), using RT-PCR, we identified THR mRNA sequences in skeletal muscles of P. anguinus which did not seem to be upregulated by T3 treatment in opposition to that observed in the TH-responsive Urodelan amphibians.

In this work, first we cloned and sequenced the 3' region of a cDNA corresponding to a thyroid hormone receptor α1 from muscles of P. anguinus. Second, using a sensitive RT-PCR method and in situ hybridization, we analyzed the THR α transcripts from different tissues of P. anguinus. We showed a tissue-specific expression of thyroid hormone receptor α genes which was never up-regulated by thyroid hormone in contrast to that observed in TH-responsive amphibians (this paper; Yaoita and Brown, 1990; Ho Huynh et al., 1993).

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Results

Using a cDNA-PCR strategy (Fig. 1), from total RNA of *P. anguinus* muscles, we cloned a partial THR cDNA of 993bp including the hormone binding domain (HBD) and the 3' untranslated region (Fig. 2). In comparison with the THR α1 cDNA of *Xenopus* (Brooks et al., 1989), this cDNA sequence showed a great similarity in coding region since only two nucleotide changes were observed: a T is replaced by a C at position 954 (position 38 of the *P. anguinus* THR cDNA), and a G instead of an A at position 1434 (position 518 of the *P. anguinus* THR cDNA). The two substitutions are conservative and do not introduce an amino acid change. The 3' untranslated sequence appeared not conserved in comparison to *Xenopus* THR α1 sequence. The high homology between the 3' coding sequence of this *Proteus* THR cDNA and that of the *Xenopus* THR α1 cDNA clearly showed that *P. anguinus* muscle contains THR α 1 subtype transcripts.

The amphibian THR mRNAs are important in size (about 10kb in *Xenopus laevis*), they are rare and difficult to detect by traditional Northern blot analysis (Yaoita and Brown, 1990). To identify THR α mRNA in tissues of the perennibranch *P. anguinus* and

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**Fig. 1. Xenopus THR α1 cDNA clone (Brooks et al., 1989) and amplification strategy used for P. anguinus THR α cDNA cloning.** The orientation of the PCR primers is shown by arrows. Oligomer sequences are noted in Materials and Methods. PCR product size of 716bp is the expected result of PCR with primers 5′/6. PCR amplification using primers 3′/UAP produced a fragment of 529bp.

**Fig. 2. Nucleotide sequence of P. anguinus muscle thyroid hormone receptor α1 cDNA reconstituted from the two cloned fragments.** (*) designates the difference observed between the P. anguinus THR sequence determined in the present study and the published sequence of *X. laevis* THR α1 (Brooks et al., 1989).
analyze their regulation by thyroid hormone, we used a sensitive cDNA-PCR assay capable of detecting small amounts of RNA (Chelly et al., 1990). The transcripts analyzed were co-reverse transcribed and co-amplified in the same reaction with an exogenous cRNA which serves as internal control. We checked that the analysis was performed in the exponential phase of amplification since the amount of amplified fragments is at that time proportional to the initial amounts of transcripts and so permits semi-quantitative comparisons (Chelly et al., 1990). The oligonucleotide pair 1/6 used for PCR amplification preferentially recognized THR α but not THR β coding sequences since the two THR genes were known to diverge in the regions corresponding to these sequences in the Xenopus, human, rat and chicken THR genes (Banker et al., 1991). The fact that there was no amplification in the absence of reverse transcriptase demonstrated that mRNA, and not genomic DNA, was amplified (Fig. 3).

Total RNA from five different tissues (brain, muscle, skin, liver and intestine) of P. anguinus were analyzed for THR α expression. PCR amplification using the 1/6 oligonucleotide pair as primers, detected for all the tissues the predicted fragment of 294 bp (Figs. 3, 4) with important level variations depending on the type of tissue when probing with cloned THR α cDNA (Fig. 4). It appeared that brain, intestine and liver were characterized by an important level of THR α mRNAs in opposition to muscle and skin, where a low level of THR α mRNAs was detected. These results were confirmed by in situ hybridization using (p33)-cRNA probes on transverse sections, which permitted the analysis of spatial localization of THR α mRNAs (Figs. 5, 6). The cRNA probes used were synthesized from the high Proteus-specific 3'terminus cDNA (32/UAP). A strong hybridization signal was observed in liver, intestinal epithelium, brain and spinal cord while muscle and skin showed a weak hybridization signal (Figs 5, 6). In our experiments, using cDNA-PCR and in situ hybridization, we showed that T3 treatment did not reveal upregulation of P. anguinus THR α mRNAs in contrast to that observed in Xenopus laevis used as control (Figs. 6, 7).

**Fig. 3.** Analysis on 1% agarose gel followed by ethidium bromide staining of the PCR fragments obtained with the primer pair 1/6 from cDNAs after reverse transcription of total RNA extracted from muscles of Xenopus tadpoles (b) used as control and Proteus anguinus (d). HaeIII-digested phi X174 DNA size standards were run in parallel (a). PCR amplification of total RNA extracted from muscles of Proteus anguinus without reverse transcription (c).

**Fig. 4.** Detection by cDNA-PCR amplification of THR α transcripts from different tissues of P. anguinus. Southern blots were performed on cDNA-PCR co-amplified products of THR α (fragment 1/6) and internal standard (fragment A/B) and hybridized with Xenopus THR α probe (upper bands). The filters were rehybridized with the radioactive oligonucleotide B (lower bands). Skeletal muscles of X. laevis tadpoles (a) used as control; skeletal muscles (b), skin (c), liver (d) intestine (e) and brain (f) of P. anguinus.

### Discussion

This report shows for the first time the presence of THR α mRNAs in a perennibranchiate species, P. anguinus, which is insensitive to massive doses of TH. It demonstrates that the low receptiveness of the perennibranch tissues to thyroid hormone was not due to a lack of THR mRNAs as previously suggested (Yaolta and Brown, 1990).

The wide expression of THR α mRNAs has been reported in different species (Banker et al., 1991; Forrest et al., 1991; Kawahara et al., 1991). In P. anguinus, THR α mRNAs were detected in all the tissues analyzed. However, important variations in the level of THR α mRNAs, depending on the type of tissue, were shown (high levels in brain, liver, intestinal epithelium; low levels in skin, muscle). Our results are in agreement with those of Kawahara et al. (1991) in X. laevis larvae, since these authors reported by in situ hybridization a high signal strength in the central nervous system, liver and intestinal epithelium and a moderate signal strength in muscle which accounts for a tissue-specific expression of the thyroid hormone receptor α genes.

Different subtypes of THRα: α1 and α2, have been characterized: THRα1 binds T3 and can act as a functional THR, while THRα2 does not bind T3 and cannot transactivate TH-responsive genes (Mitsuhashi et al., 1988; Koenig et al., 1989; Mitsuhashi and Nikodem, 1989). THRα2 might act as an inhibitor of thyroid hormone action by mechanisms still unknown (Lazar, 1993). Analysis of the 3'terminus coding sequence clearly showed that the THR mRNAs detected in P. anguinus were of the subtype α1, coding for a THR protein capable of binding T3 in all the species analyzed (Mitsuhashi et al., 1988). Nevertheless, we have presented no evidence that THRα1 is expressed in tissues of P. anguinus since our analysis only mea-
sured mRNA levels, not the proteins that they encode, and we cannot exclude a possible non-expression of the THRα protein. However, the report of Galton and Germain (1985) supports the idea of the presence of THR protein in perennibranchiate species. Following this hypothesis, we have to take a problem into consideration: is the *P. anguinus* THRα1 mRNA intact and full-length able to give a functional protein? In particular, is its DNA-binding domain intact? It is interesting to note that, in man, in all cases of generalized thyroid hormone resistance, which is a syndrome of hyposensitivity to T3, it appears that the mutant THR displays abnormal T3-binding but always normal DNA-binding activities (reviewed in Yen and Chin, 1994).

It has been clearly shown that T3 regulates the THR mRNAs levels in different species. In *Xenopus*, experimental hyperthyroidism showed that exogenous T3 can upregulate THRβ mRNA as much as 20-fold during tadpole development, whereas TH

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**Fig. 5.** Transversal troncal sections of *P. anguinus* hybridized with *P. anguinus* THR α1 antisense (A) and sense RNA (B). Sc, spinal cord; M, muscle; S, skin; I, intestine; L, liver. Note that skin produces an artefactual signal with sense RNA. Autoradiograms exposed for 15 and 30 days for antisense and sense probes, respectively.

**Fig. 6.** In situ hybridization using *P. anguinus* THR α1 antisense RNA on transversal sections of *P. anguinus* brain (A,B) and muscle (C,D). Control (A,C) and T3 treated animals (B,D). Autoradiograms exposed for 1 week (A,B) or for 15 days (C,D).
upregulates THRα mRNA by 2-fold during the same period (Yaoita and Brown, 1990). For THRα mRNA, the same period have been obtained for Rana esculenta (Schneider and Galton, 1991) and TH-sensitive Urodela amphibians using cDNA-PCR assay (Ho Huynh et al., 1993). We clearly showed that T3 treatment did not reveal upregulation of P. anguinus THR α mRNAs in all the tissues analyzed. The formation of T3-bound THR complexes specifically interacting with thyroid hormone-response elements (TREs) located in regulatory regions of target genes is a necessary first step for activation or suppression of target genes (Yen and Chin, 1994). It has been demonstrated that the promoter of the Xenopus THRβ gene contains a TRE which would account for its T3 induction (Ranjane et al., 1994). In contrast, the presence of TRE in the promoter of the THR α gene is not clear. Ishida et al. (1993) did not detect TRE in the human RHTα promoter region and suggested that the expression of RHTα might be indirectly regulated by T3. Currently we do not have any information on the structure of the THRα1 promoter in P. anguinus. Nevertheless, the TH-insensitivity of the perennibranchiate P. anguinus should be correlated with the inability of exogenous T3 to upregulate THRα mRNAs in this species in opposition to that shown in all the TH-sensitive species. An interesting way of exploring a better understanding of the TH-insensitivity of perennibranch amphibians could be the analysis of the prolactin level in P. anguinus. Moreover, it appearsthatprolactin preventstherapidT3-suppressiveeffectinP. anguinus.

Materials and Methods

Animals

Xenopus laevis tadpoles originated from the laboratory stock. All animals were kept in tap water at 18°C. Individuals from the species Proteus anguinus belonged to a stock that has been established in the CNRS cave laboratory at Moulis in France since 1952. Animals were raised under semi-natural conditions in stream water at 10°C and in the dark. In experimental hyperthyroidism procedures, animals were treated with 3.5,3'-triiodothyronine (T3); water in the breeding tanks was replenished with T3 (5x10⁻⁵ M) and this medium was changed every day. T3 treatment was continued for 3 weeks.

Purification and quantification of total RNAs

Total RNA was purified by the method of Auffray and Rougeon (1980) and was checked by agarose gel electrophoresis and ethidium bromide staining. The amount of total RNA used in the cDNA-PCR amplification was controlled by hybridization with a radioactive 24-mer oligonucleotide complementary to the rat 18S ribosomal RNA (Mercadier and Dubus, 1991).

cDNA-PCR amplification

First-strand cDNA synthesis

First-strand cDNA was synthesized from 5 μg of total RNA of each tissue. An exogenous sample of cRNA (about 1 pg) transcriptional product of the pGEM-5Zf poly linker region (Promega) was added as internal standard RNA for expression studies. MMLV reverse transcriptase (Pharmacia) was used to produce first-strand cDNAs in 20 μl reaction volume containing 50 mM KCl, 10 mM Tris (pH 8.3), 16 mM MgCl₂, 1 U of RNasin (Promega) per μl, 500 μM deoxynucleoside triphosphates, 50 pmol of random primers (Gibco BRL) and internal standard-specific oligonucleotide B. Reactions were performed at 37°C for 2 h, stopped at 95°C for 5 min, and held on ice. First-strand cDNA for 3'end amplification was performed with an adapter primer provided by the 3'RACE (Rapid Amplification of cDNA Ends) System (Gibco BRL) kit.

PCR amplification

3 μl of reverse transcription reaction mix was added to a PCR reaction mix containing the buffer described above. 2 U of Taq polymerase (Perkin-Elmer Cetus), 50 pmol of each primer, and a final dNTP concentration of 200 μM. Amplification strategy for THR α cDNA cloning is shown in Figure 1.

In the first step, amplification was performed by using Xenopus THR α oligonucleotides 5'-8 which are highly conserved among species. In the second step, sense primers were selected for 3'region (31 and 32) amplification. Antisense oligonucleotide (UAP) for 3'terminal amplification was an universal primer provided with the 3'RACE System (Gibco BRL).

On the basis of the sequence of the Xenopus THR α cDNA clone (Brooks et al., 1989), position of the oligonucleotide primers used are as follows:

- OI 8: 5'-GGGCGAATTGGGCCCGACGT-3'
- OI 31: 5'-GGGAGCAGCTTIAAGAACGC-3'
- OI 32: 5'-CAUCAUCACAUCAUCATTGCGGACC-3'
- CI 5: 5'-GGGAGAAGGGAGGACGAGTATGTCG-3'

For the studies of THR α expression, sense oligonucleotide γ (5'-GACCTCAAGCCTGAGCCG-3') and antisense oligonucleotide 6 (5'-CTCTACGAGCGATTTGAGG-3') were used and produced a fragment of 294bp. On the basis of the sequence of the pGEM-5ZI polylinker region, the two oligonucleotides used for amplification of internal control were (5'-GGGCGAATTGGGCCCGACGT-3') as sense (oligonucleotide A) and (5'-GGGCGAATTGGGCCCGACGT-3') as antisense primer (oligonucleotide B), and produced a 111bp fragment.

25 cycles of PCR were performed using a thermocycler with cycles consisting of 40 s of denaturation at 94°C, 1 min of annealing
at 52°C, and 1 min of extension at 72°C. PCR products were ana-
lyzed by separation on 7% polyacrylamide gels followed by ethidium bromide staining and by Southern blotting and probing with radioac-
tive Xenopus THR α probes.

Cloning and sequencing of PCR fragments
The fragment amplified from muscles using the primer pairs 5′/8 was
first purified by Gene clean kit (Bio 101), blunted with T4 DNA poly-
merase (Boehringer Mannheim Biochimca), and phosphorylated with
T4 Polynucleotide Kinase (Gibco BRL). The treated fragments were
then subcloned in Sma I site of pGEM-4Z Vector (Promega). Amplified frag-
ments of 3′ terminus were cloned in psPORT 1 vector provided by the
CLONEAMP System (Gibco BRL) kit, using the sequence CAU-CAU-
CAU-GAU of both. of 3′/2 and UAP. Double-stranded DNA was sequenced
using T7 Sequencing kit (Pharmacia) and [35S]dATP (NEN Research Product).

In situ hybridization
The procedure for fixing, embedding and sectioning tissues was as
for mouse embryos and, as with the procedure for in situ hybridization,
was essentially as described by Wilkinson and Green (1990). Briefly,
tissues were fixed in 4% paraformaldehyde in PBS, dehydrated and
infiltrated with paraffin, 6 μm thick, serial sections were mounted on
TESPA-coated RNase-free glass slides. Sections were deparaffinized
in xylene, treated with triethanolamine/acetic anhydride, washed and
dehydrated. High-stringency conditions for hybridization were followed
with post-hybridization washing in 2xSSC, 50% formamide, 50 mM DTT
at 65°C for 30 min. Autoradiography was carried out with Kodak NTB-2
track emulsion and developed in Kodak 019 developer. psPORT 1 vec-
tor containing the 3′terminus of P. anguinus THR α cDNA was lin-
erized by EcoR1 and (P33)-cRNA probes were synthesized using SP6
RNA Polymerase. Probes were hydrolyzed to an average of 120
nucleotides.

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