Int. J. Dev. Biol. 40: 537-543 (1996)

Original Article

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 hypothyroidian *A. mexicanum*, where metamorphosis does not occur spontaneously, this transition is partial; larval and fast isomyosins are coexpressed in adult muscles. Experimental hyperthyroidism involves anatomical metamorphosis and a complete transition from larval to fast isomyosins 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).

Abbreviations used in this paper: RT, reverse transcriptase; PCR, polymerase chain reaction; THR, thyroid hormone receptors; TH, thyroid hormone.

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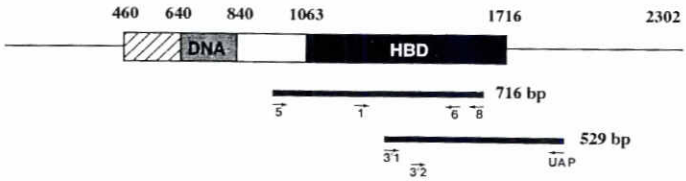

Fig. 1. *Xenopus* THR α1 cDNA clone (Brooks *et al.*, 1989) and amplification strategy used for *P. anguinus* THR α cDNA cloning. *The orientation of the PCR primers is shown by arrows. Oligomer sequences are noted in Materials and Methods. PCR product size of 716bp is the expected result of PCR with primers 5/8. PCR amplification using primers 3/UAP produced a fragment of 529bp.*

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 mRNAs in tissues of the perennibranch *P. anguinus* and

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GG CGG AAG GAG GAG ATG ATC AAG ACT CTG CAA CAG CGC CCC GAG CCA 47
AGC AGC GAG GAG TGG GAG TTG ATT CGC ATT GTA ACA GAA GCT CAC AGG 95
AGT ACC AAT GCT CAG GGC AGC CAC TGG AAA CAG CGT AGG AAG TTT CTG 143
CCG GAA GAT ATC GGG CAG TCT CCC ATG GCT TCC ATG CCG GAT GGG GAT 191
AAA GTT GAC CTG GAA GCT TTC AGT GAG TTC ACC AAG ATA ATC ACC CCG 239
GCA ATT ACC AGA GTG GTG GAC TTT GCC AAG AAG CTG CCC ATG TTC TCT 287
GAG CTG ACT TGT GAA GAC CAG ATC ATC CTG TTG AAA GGA TGT TGT ATG 335
GAG ATC ATG TCT CTC CGT GCT GCT GTA CGC TAC GAT CCA GAC AGC GAG 383
ACC CTA ACG CTG AGC GGA GAG ATG GCT GTG AAA CGG GAG CAG CTT AAG 431
AAC GGA GGT CTG GGT GTT GTC TCT GAT GCC ATC TTT GAC CTC GGG AGG 479
TCG CTT GCT GCG TTT AAC CTT GAC GAT ACG GAA GTG GCG CTG CTG CAG 527
GCT GTT TTG CTA ATG TCA TCA GAC CGA ACT GGT TTA ATC TGC ACG GAC 575
AAG ATA GAG AAA TGT CAA GAG ACC TAC CTT CTC GCC TTT GAA CAC TAC 623
ATC AAC CAT CGC AAA CAC AAC ATT CCC CAC TTC TGG CCC AAA CTC CTA 671
ATG AAG GTG ACG GAC CTG CGC ATG ATA GGG GCA TGC CAT GCC AGC CGC 719
TTT CTG CAC ATG AAG GTC GAG TGC CCC ACC GAG CTC TTT CCA CCG CCT 767
TTC CTT GAG GTC TTT GAG GAC CAG GAA GTT TGA GGGAACCTGCGTGCTGGTA 819
GGGAGGCGAAAGCAAACTAAGCTTTGATTCACGCCTAACTAGGTACGCAATTCTATCCCAAGA 882
AGACAATCACTGATCAAGGAGCTACAGAAAACCGTAACTTTATTATAGGTACTCCGAGAAATG 945
GAAGTTATCAAGAGATGTTCTAAGTAAAGCAAAAAAAAAAAAAAAAAAA 993
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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 transcripted 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 tissues when probing with cloned THR α cDNA (Fig. 4). It appeared that brain, intestine and liver were characterized by an important level of THRα 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 (P^{33})-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 (3'2/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).**

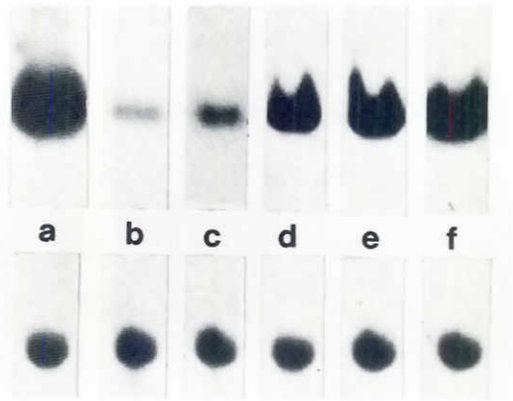

Fig. 4. Detection by cDNA-PCR amplification of THR α transcripts from different tissues of *P. anguinus*. *Southern blots were performed on cDNA-PCR co-amplified products of THR α (fragment 1/6) and internal standard (fragment A/B) and hybridized with Xenopus THR α probe (upper bands). The filters were 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 (Yaoita 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 THRα 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* tissues 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-

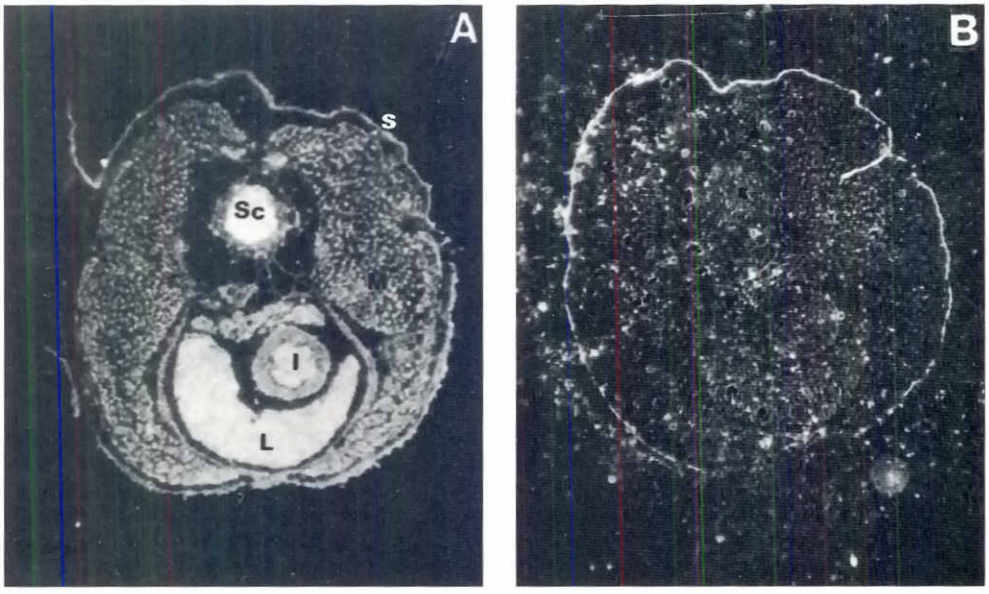

Fig. 5. Transversal troncal sections of *P. anguinus* hybridized with *P. anguinus* THR α1 antisense (A) and sense RNA (B). *Sc, spinal cord; M, muscle; S, skin; I, intestine; L, liver. Note that skin produces an artefactual signal with sense RNA. Autoradiograms exposed for 15 and 30 days for antisense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

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

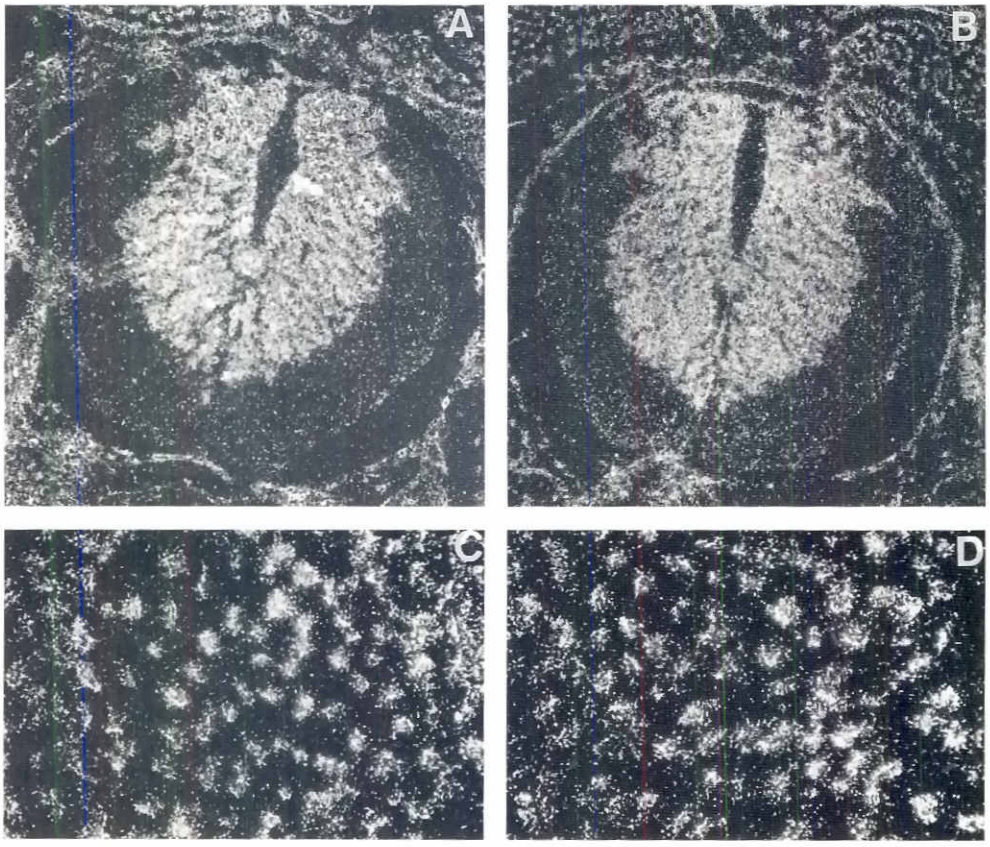

Fig. 6. *In situ* hybridization using *P. anguinus* THR α1 antisense RNA on transversal sections of *P. anguinus* brain (A,B) and muscle (C,D). *Control* **(A,C)** *and T3 treated animals* **(B,D)**. *Autoradiograms exposed for 1 week* **(A,B)** *or for 15 days* **(C,D)**.

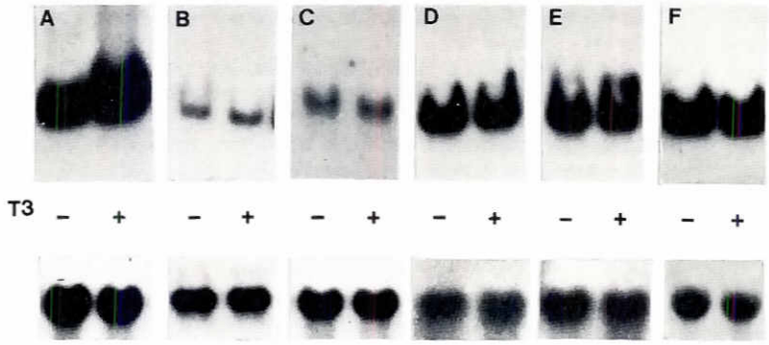

Fig. 7. Detection and relative quantification by cDNA-PCR amplification of THR α transcripts from different tissues of *P. anguinus* following T3 treatment. *Southern blots were performed on cDNA-PCR co-amplified products of THR α (fragment 1/6) and internal standard (fragment A/B) and hybridized with 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.*

upregulates THRα mRNA by 2-fold during the same period (Yaoita and Brown, 1990). For THRα mRNA, the same results have been obtained for *Rana esculenta* (Schneider and Galton, 1991) and TH-sensitive Urodelan 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 (Ranjan *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-insensivity of perennibranch amphibians could be the analysis of the prolactin level in *P. anguinus* since prolactin blocks the precocious induction of metamorphosis by exogenous T3 (Tata *et al.*, 1991). Moreover, it appears that prolactin prevents the rapid T3-induced upregulation of THRα and THRβ mRNAs in *Xenopus* tadpoles and in organ cultures of tadpole tails (Baker and Tata, 1992).

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 complemented with T3 ($5x10^{-8}$ 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 controled by hybridization with a radioactive 24-mer oligonucleotide complementary to the sequence of 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 1pg), transcriptional product of the pGEM-5Zf polylinker 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 $MgCl_2$, 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 (3'1 and 3'2) amplification. Antisense oligonucleotide (UAP) for 3'terminus amplification was an universal primer provided with the 3'RACE System (Gibco BRL).

On the basis of the sequence of the *Xenopus* THR α1 cDNA clone (Brooks *et al.*, 1989), position of the oligonucleotide primers used are as followed :

Ol 8: 5'-GCTGGCATGGCATGCCCC^{1615}-3'.
Ol 3'1: 5'-CGGGAGCAGCTTAAGAACGG^{1352}-3'
Ol 3'2: 5'-CAUCAUCAUCAUGCCATCTTTGACCTCGGGAGG^{1395}-3'
Ol 5: 5'-GGCGGAAGGAGGAGATGATC^{936}-3'.

For the studies of THR α expression, sense oligonucleotide γ (5'-GACCCTAACGCTGAGCGG^{1316}-3') and antisense oligonucleotide 6 (5'-CTTCATTAGGAGTTTGGG^{1576}-3') were used and produced a fragment of 294bp. On the basis of the sequence of the pGEM-5Zf polylinker region, the two oligonucleotides used for amplification of internal control were (5'-GGGCGAATTGGGCCCGACGT-3') as sense (oligonucleotide A) and (5'-GCATCCAACGCGTTGGGAGC-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 analyzed by separation on 7% polyacrylamide gels followed by ethidium bromide staining and by Southern blotting and probing with radioactive *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 polymerase (Boehringer Mannheim Biochemica), and phosphorylated with T4 Polynucleotide Kinase (Gibco BRL). The treated fragments were then subcloned in *Sma I* site of pGEM-4Z Vector (Promega). Amplified fragments of 3'terminus were cloned in pSPORT 1 vector provided by the CLONEAMP System (Gibco BRL) kit, using the sequence CAU-CAU-CAU-CAU of both, ol 3'2 and UAP. Double-stranded DNA was sequenced using T7 Sequencing kit (Pharmacia) and [^{35}S] 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 um 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 D19 developer. pSPORT 1 vector containing the 3'terminus of *P. anguinus* THR α cDNA was linearized by EcoR1 and (P^{33})-cRNA probes were synthesized using SP6 RNA Polymerase. Probes were hydrolyzed to an average of 120 nucleotides.

Acknowledgments

This work was supported by grants from the Centre National de la Recherche Scientifique and the Association Française contre les Myopathies. We are most grateful to Dr. D.D. Brown, Carnegie Institution of Washington, Baltimore, for giving us the Xenopus thyroid hormone receptor α cDNAs. We also wish to thank Dr. Christian Juberthie, Director of the Moulis Cave Laboratory (CNRS) for the gift of P. anguinus.

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Received: September 1995
Accepted for publication: March 1996