Identification of Thyroid Hormone Receptor Transcripts in Muscle of a Perennibranch Amphibian (*Proteus Anguinus*)

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Abstract

The perennibranch amphibian, *Proteus anguinus*, is insensitive to massive doses of thyroid hormone. It never undergoes anatomical metamorphosis in contrast to what happen in the TH-sensitive amphibian species. In this report, using a sensitive cDNA-PCR assay, we detected thyroid hormone receptors mRNAs in the skeletal muscles of the four amphibian species analysed. In *P. anguinus*, these THR mRNAs were not up regulated by T3 treatment in opposition to that observed in the TH-sensitive species *(Xenopus laevis, Pleurodeles waltlii* and *Ambystoma mexicanum)*. We noted a correlation between the ability to carry out a complete larval-to-fast myosin heavy chain transition in natural *(P. waltlii, X. laevis)* or experimental *(A. mexicanum)* conditions and the ability of exogenous T3 to up-regulate THR mRNAs. *P. anguinus*, where a complete larval-to-fast myosin heavy chain transition was never observed, did not present up-regulation of THR mRNAs by exogenous T3.

Key words: thyroid hormone receptors, perennibranch amphibian, *Proteus anguinus*, amphibian muscle.

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Amphibian metamorphosis is a complex biological event which totally depend on the presence of thyroid hormone [9]. It acts via a nuclear family of thyroid hormone receptors which modulates the transcription of thyroid hormone-responsive genes [6, 8, 10]. Some salamanders, described as "obligate neotenes" never undergo metamorphosis. They are insensitive to massive doses of thyroid hormone. However, they possess thyroid glands, the products of which can induce metamorphosis in other species [7]. In amphibians, muscle development is characterized by a larval to fast myosin heavy chain transition regulated by thyroid hormone during anatomical metamorphosis [4]. In the hypothyroidian *A. mexicanum*, where metamorphosis does not occur spontaneously, this transition is partial; the larval and fast isomyosins coexisting in adult muscles. Experimental hyperthyroidism involve anatomical metamorphosis and a complete transition from larval to fast isomyosins in this species. In the perennibranch *P. anguinus*, there is also a partial myosin isoform transition but long-term T3 treatment fail to involve a complete transition [5]. In this connection, [12] noted that *P. anguinus* skin did not respond to thyroxine (T4). More recently, it has been suggested that the low receptiveness of the perennibranch tissue to the thyroid hormone could be due to a lack of THR mRNAs [16]. In the view of a better understanding of the regulation by TH of gene expression in amphibian muscle, using a sensitive cDNA-PCR method, we analyse THR transcripts extracted from muscles of *P. anguinus* in comparison to those from muscles of two TH-responsive urodeles, *P. waltlii*, where metamorphosis occurs spontaneously and the "facultative neotene" *A. mexicanum*.

Materials and Methods

Animals

Xenopus laevis tadpoles and larvae of the two urodelan species, *Pleurodeles waltlii*, and *Ambystoma mexicanum* 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. All animals were treated with 3, 5, 3'-triiodothyronine (T3); water in the

breeding tanks was complemented with T3 (5. 10^{-8} M) and this medium was changed everyday. T3 treatment was continued for 3 weeks.

Purification and quantification of total RNAs

Total RNA was purified by the method of [1] 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 hydridization with a radioactive 24-mer oligonucleotide complementary to the sequence of the rat ribosomal RNA [13].

cDNA-PCR amplification

First-strand cDNA synthesis

To 5 µg of total RNA of each specimen, an exogenous sample of cRNA (about 1 pg), transcriptional product of the polylinker region of pGEM-5Zf (Promega), was added as internal standard RNA. MMLV reverse transcriptase (Pharmacia) was used to produce first-strand cDNAs from RNAs 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 deoxynucleosides triphosphates, 50 pmol of each forward primer corresponding to the 3' part of the fragments to be amplified: THR -specific oligonucleotide 8 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.

PCR amplification

3 µl of this 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. For THR alpha, the 5' (sense) primer used was designated as oligonucleotide 1. It sequence is 5'-GACCCTAACGCTGAGCGG-3' as at nucleotide position 839 of the protein-coding sequence of the Xenopus THR cDNA [2]. The 3' (antisense) primers are designated oligonucleotide 6, which corresponds to nucleotide position 1116 (5'-CCCAAACTCCTAATGAAG-3'), and oligonucleotide 8, which correspond to nucleotide position 1155 (5'-GCTGGCATGGCATGCCCC-3'). On the basis of the positions of the oligonucleotides sequences in *Xenopus* cDNA clone sequence, the size of PCR fragments obtained with these primers could be predicted: the primer pair including oligonucleotides 1 and 6 (hereafter called 1/6) produced fragment of 294 bp from cDNA while products of the primer pair including oligonucleotides 1 and 8 (hereafter called 1/8) was a 333 bp fragment (Fig. 1A).

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 111 pb 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 analysed by separation on 7% polyacrylamide gels followed by ethidium bromide staining and by Southern blotting and probing with radioactive *Xenopus* THR alpha probes.

Results

The amphibian THR mRNAs are important in size (about 10 kb in *Xenopus laevis)*, they are rare and difficult to detect by traditional Northern blot analysis [16]. To identify THR mRNAs in the perennibranch *P. anguinus* and analyse their regulation by thyroid hormone we used an extremely sensitive cDNA-PCR assay able to detect small RNA amounts [3]. The analysed transcripts were co-reverse transcripted and co-amplified in the same reaction with an exogenous cRNA which serve of internal control. We checked that the analysis was performed in the exponential phase of amplification (data not shown) since the amount of amplified fragments is at that time proportional to the initial amounts of transcripts [3] and so permitted semi-quantitative comparisons. Total RNA from skeletal muscles and some other tissue of the three urodelan amphibian species: *P. waltlii, A. mexicanum* and *P. anguinus* and of *X. laevis* tadpoles used as control, were analysed for THR alpha expression. PCR amplification using the 1/6 or 1/8 *Xenopus* oligonucleotide pair as primers detected for all the species the predicted fragments of 294 bp (Fig. 1B) or of 333 bp (data not shown) respectively, when probing with cloned Xenopus THR alpha cDNA.

In *X. laevis* tadpoles, T3 treatment showed two-fold up-regulation of THR alpha mRNA in accordance to [16]. We measured the levels of THR mRNAs in extracts of muscles of the three urodelan species, *P. waltlii, A. mexicanum* and *P. anguinus* as a response to exogenous T3 treatment. We showed up-regulation of *P. waltlii* and *A. mexicanum* THR mRNAs of about two fold following T3 treatment as observed in *X. laevis*. In opposition T3 treatment did not reveal up-regulation of *P. anguinus* THR mRNAs (Fig. 2).

Discussion

In X. laevis, the members of the two known thyroid hormone receptor (THR) families alpha and beta have been cloned and characterized [11, 15, 17]. The 1/6 and 1/8 oligonucleotides pairs preferentially recognize THR alpha but not THR beta 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 [2]. In *Xenopus* and *Rana esculenta,* previous works showed that exogenous TH up-regulated THR beta and THR alpha mRNAs [11, 14, 16]. The cDNA-PCR method that we have used in our experiments can yield data analogous to those obtained by using more traditional assays of RNA expression since we showed a two-fold up-regulation of THR alpha mRNAs following T3 treatment in *Xenopus* tadpoles in accordance to the results of [16]. In the absence of sequence information about THR genes in urodelan amphibians, we have used the two *Xenopus* oligonucleotides pairs in our cDNA-PCR assay and always obtained the predicted fragments showing

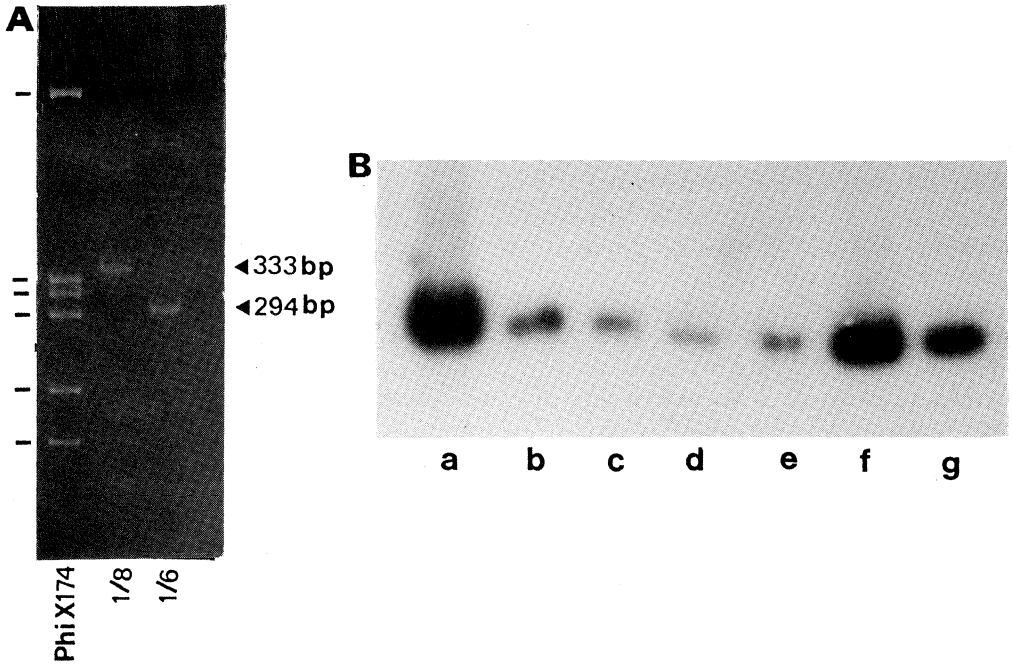

Figure 1. (A) Analysis on 7% polyacrylamide gel followed by ethidium bromide staining of the PCR fragments obtained with the primer pair 1/8 and 1/6 from cDNAs after reverse transcription of total RNA extracted from muscles of Xenopus tadpoles. HaeIII-digested phi X174 DNA size standards were run in parallel as shown. (B) detection by cDNA-PCR amplification of THR transcripts using Xenopus primer pair 1/6 in different amphibian tissues. Skeletal muscles of X. laevis tadpoles(a), P. waltlii (b), A. mexicanum (c) and P. anguinus (d); skin of P. anguinus (e) and skin (f) and gills (g) of A. mexicanum. The PCR fragments were analysed by Southern blotting and hybridization with Xenopus THR alpha probe.

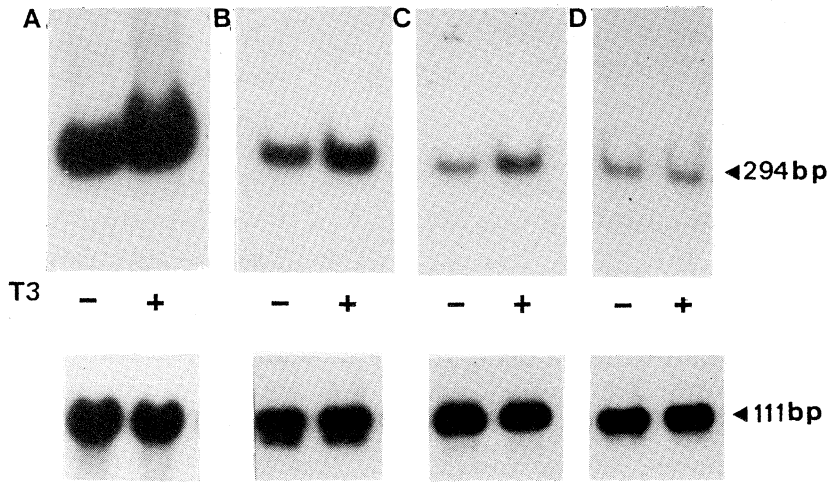

Figure 2. Detection and relative quantification by cDNA-PCR amplification of THR transcripts in skeletal muscles of different amphibians following T3 treatment. Southern blots were performed on cDNA-PCR co-amplified products of THR (fragment 1/6) and internal standard (fragment A/B) and hybridized with Xenopus THR alpha probes (upper bands). The filters were rehybridized with the radioactive oligonucleotide B (lower bands). (A) X. laevis, (B) P. waltlii, (C) A. mexicanum, (D) P. anguinus.

substantial hybridization with the *Xenopus* THR alpha probes. It should be noted that in *A. mexicanum*, the *Xenopus* THR probes did not distinguish the THR alpha and THR beta gene family in the heterologous hybridation reactions [16]. However, the use of specific THR alpha primers and the two-fold up-regulation of THR mRNAs after T3 treatment observed in *P. waltlii* and *A. mexicanum* suggested that the amplified products represented THR

alpha sequences since it appeared that THR beta mRNAs showed a more important up regulation by TH [11, 16]. This report show for the first time the presence of THR mRNAs in a perennibranch species, *P. anguinus*, which is insensitive to massive dose of TH. This TH- insensitivity should be correlated to the unability of exogenous T3 to up-regulate THR mRNAs in this species in opposition to the two TH-sensitive species, *P. waltlii* and *A. mexicanum* where exogenous T3 up-regulate THR mRNAs.

Previous works showed that TH regulated the larval-to-fast myosin heavy chain transition [4, 5] but there is no clear evidence that the action of TH is direct on fast MHC expression. However, we noted a correlation between the ability to carry out a complete larval-to-fast MHC transition in natural *(P. waltlii)* or experimental *(A. mexicanum)* conditions and the ability to exogenous T3 to up-regulate THR mRNAs. *P. anguinus* where a complete larval-to-fast MHC transition was never observed, did not present up-regulation of THR mRNAs by exogenous T3.

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Abbreviations: PCR, polymerase chain reaction; THR, thyroid hormone receptors; TH, thyroid hormone; MHC, myosin heavy chain