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Short Contribution

Thyroid hormone receptors in perennibranchiate amphibians

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ABSTRACT Thyroid hormone has long been known to induce metamorphosis in amphibians. The understanding of the molecular steps controlling the completion of metamorphosis has nevertheless been hampered by the complexity of this event. The comparison of organisms in which metamorphosis does or does not occur, may provide clues into the molecular cascade that control it. Up to now the available data suggest that perennibranchiate amphibians retain their larval characters mainly because their tissues do not respond to thyroid hormones. In such a context the recent identification of a thyroid hormone receptor α in the perennibranchiate *Proteus anguinus* is provocative (Ho Huynh *et al., Int. J. Dev. Biol. 40:* 537-543, 1996). In the present paper, we provide evidences that this recently described sequence is in fact a sequence from *Xenopus laevis*. Indeed, we identified the authentic thyroid hormone receptors of both α and β types in two perennibranchiate species *Necturus maculosus* and *Proteus anguinus*. The various controls required to ascertain the authenticity of a developmental gene cloned by PCR or RT-PCR analysis are presented. The results reported in the present paper are relevant with phylogenetical analysis. This induces our team to conclude that the *Proteus* TRα sequence reported by Ho Huynh *et al.* (1996) reflects a contamination of the RT-PCR by *Xenopus laevis* material.

KEY WORDS: *thyroid hormone receptors, amphibians, metamorphosis, PCR*

Thyroid hormones and their receptors play a pivotal role in amphibian metamorphosis (Gilbert *et al.*, 1996). To better understand this complex physiological process, it is worth studying organisms in which metamorphosis does not occur. Such an approach will allow us to compare neotenic animals with their metamorphosing relatives. The Proteidea family comprises obligatory neotenic species such as the American mudpuppy *Necturus maculosus* and a European species *Proteus anguinus* (Turner and Bagnara, 1976; Bentley, 1982; Shaffer, 1993). In paedomorphs, a major research theme has been to determine the cause of the interruption in the metamorphosis cascade. *Necturus* and related species (Proteidae, Hedges and Maxson, 1993) clearly have a functional thyroid gland, yet even large doses of thyroid hormones fail to produce any morphological change (reviewed in Turner and Bagnara, 1976; Gilbert and Frieden, 1981). Thus, it seems particularly interesting to isolate thyroid hormone receptor genes in *Necturus* and *Proteus* in order to scrutinize their molecular characteristics in relation with the absence of metamorphosis. Recently, Ho Huynh *et al.* (1996) have reported the cloning and characterization of a thyroid hormone receptor α1 in *Proteus anguinus*. By RT-PCR and *in situ* hybridization experiments, these authors claimed that TRα expression is tissue-specific and is not regulated by thyroid hormones. Given the non-responsiveness to thyroid hormones of *Proteus* tissues the cloning of a TRα homolog appears provocative and interesting.

The examination of the published *Proteus* TRα gene sequence reveals 99.75% nucleotide identity with the *Xenopus laevis* TRα gene in the coding portion of the sequence rendering it more closely related to the latter than to the *Rana catesbeiana* one (Fig. 1A; Yaoita *et al.*, 1990; Schneider and Galton, 1991). *Proteus* is a urodele, *Xenopus* and *Rana* are two Anurans, and a phylogeny based on the analysis of the mitochondrial 12S rRNA gene clearly reveals an early divergence of Anurans and Urodeles (Fig. 1B; Hedges and Maxson, 1993). In contrast, in the paper by Ho Huynh *et al.* (1996), the so-called *Proteus* TRα sequence appears paradoxically more closely related to *Xenopus* than to *Rana*. This clearly suggests that this sequence is the result of a contamination of the RT-PCR by *Xenopus laevis* material. In the 3' untranslated region of the so-called *Proteus* TRα sequence, the low level of sequence identity (67.8%) with the *Xenopus* sequence initially described by Yaoita *et al.* (1990) is probably due to the fact that the contaminant *Xenopus* material came from a different strain than the one used in the original description. Given that the expression studies were all done with the *Xenopus* contamination artefact, the results of Ho Huynh *et al.* (1996) concerning the tissue-specific expression and the T3 regulation of TRα in *Proteus* are doubtful.

Abbreviations used in this paper: PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase PCR; T3, triiodothyronine; TRα, thyroid hormone receptor alpha; TRβ, thyroid hormone receptor beta; NJ, Neighbor-Joining method of phylogenetical tree reconstruction.

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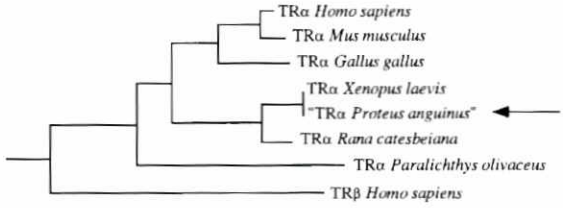
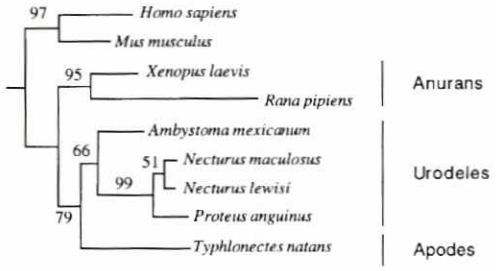
A : TR



B 12S rRNA



C : TR

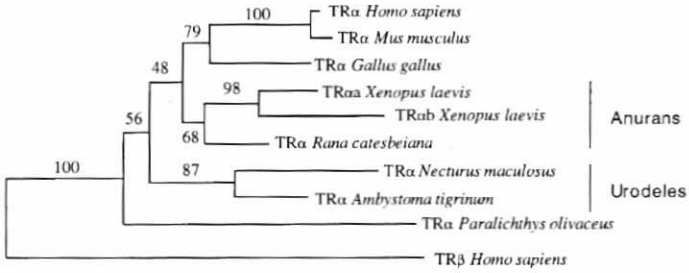


Fig. 1. (A) *Phylogenetical NJ tree (Philippe, 1993) based on the amino acid sequences of TRα genes. Only the C-term part of the so-called Proteus anguinus TRα sequenced in Ho Huynh et al. (1996) was used to calculate the tree. The position of the so-called Proteus sequence (indicated by an arrow) is obviously wrong since it should be outside of the Xenopus and Rana group (i.e. Anurans).* **(B)** *Phylogenetical NJ tree connecting partial sequences of the mitochondrial 12S rRNA gene. Only transversions were analyzed. 1000 bootstrap replicates were performed. The early split of Anurans and Urodeles is clearly observed in this tree. In fact the monophyly of amphibians is not strongly supported in the original study of Hedges and Maxson (1993).* **(C)** *Phylogenetical NJ tree with 1000 bootstrap replicates based on the amino acid sequences of the central part of the TRα sequences corresponding to the regions of the Necturus TR and Ambystoma TR that were sequenced. Although the amphibians are not monophyletic in this analysis due to the early divergence of Anurans and Urodeles, the Necturus sequence is as expected inside the Urodeles lineage.*

The definitive proof that the described *Proteus* sequence is a contamination comes from our successful cloning of TR genes in the related species *Necturus maculosus* (Safi *et al.*, 1997). By PCR and RT-PCR experiments we have isolated fragments of TRα and TRβ genes in *Necturus*. The TRα sequence that we isolated in *Necturus* harbors 88.6% and 89.7% identity at the amino acid level with the ones of *Xenopus* and *Rana* respectively. In a molecular phylogeny analysis the TRα sequence from *Necturus* is correctly located in the Urodele lineage (see Fig. 1C). We detected a strong expression of TRα in gills, intestine and liver. Importantly, PCR analysis identified TRα and TRβ sequences in *Proteus* that exhibit more identity with the *Necturus* ones than with TR sequences from any other species (Safi *et al.*, 1997; Fig. 2). Furthermore, amplification of the 12S rRNA gene in *Proteus* and *Necturus* demonstrates that our template DNA is not contaminated by foreign DNA (Fig. 1B).

It is well known that even if blank RT-PCR controls effectively yield no PCR product as in the Ho Huynh *et al.* (1996) paper, a contamination artefact may arise from a low amount of "carry-over" contaminating molecules or by a direct contamination of the tissue preparation (Kwok and Higuchi, 1989). Researchers should keep in mind that the authenticity of sequences obtained by PCR always has to be ascertained. Indeed, such a control is not obvious in the case of Proteidea since, due to the very large size of the genome, it is extremely difficult to obtain positive signals in Southern blot (Vignali and Nardi, 1996). In that respect a positive signal obtained by *in situ* hybridization technique (a method notably prone to cross-hybridization artefacts) is not a valid argument in favor of the authenticity of the sequences (Cox *et al.*, 1986 and references therein). Other methods analyzing expression such as high-stringency northern blotting or RNAase protection experiments may directly give a proof of the relevance of the sequence. Furthermore, the phylogenetical analysis of a sequence gives a strong argument for its authenticity. In this respect, the two TRα sequences that we have cloned from *Proteus* and *Necturus* are more related to each other than to any other TR sequence.

This new example shows once again the extreme importance of contamination safety procedures during the isolation of homologs of known developmental genes in various species. The use of DNA-free rooms, different laboratories for extraction and cloning/sequencing, the systematic test of possible contamination through the parallel amplification of reference sequences as well as the phylogenetical analysis of the data are prerequisites before publication of PCR-based results (Hänni *et al.*, 1994).

Experimental Procedures

Sequences

The *Homo sapiens, Mus musculus, Gallus gallus, Xenopus laevis, Rana catesbeiana, Paralichthys olivaceus* TR sequences were obtained from

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TRAH      CCTGCGGGCGGCTGTCGGCTACGACCCTGAGAGCGACACCCTGACGCTGAGTGGGGAGATGGCTGTCAAGCGGGAGCAGCTCAAGAATGGCGGCCTGGGCGTAGTCTCCGACGCC
TRAXA     T..C..T..T.....A.........T..A..C.....G.....A.........C..A...........G..A...........T.....C..A..T.....T..T.....T..T...
HO HUYNH  T..C..T..T.....A.........T..A..C.....G.....A.........C..A...........G..A...........T.....C..A..T.....T..T.....T..T...
TRAXB     T..T..T..T.....G.........T..A..C.....G.....A.........C..C.........G..G..A...........T.....C..A..T.....T..T.....T..T...
TRARAN    T..C.....T.....G..T..T..T..A..C..T..G..TT....A.....C..T.........A..G.....A..A.....G.........T..A..T..G..G.....A..T..T
TRAPRO    T.....C..C..A..G...........G..A.....G..G.....A..A..C...........G..G..............G.........T.........G..T..G..G......
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Fig. 2. Sequence comparison *of the PCR product of the bona fide Proteus TRα (TRAPRO) compared to the human TRα (TRAH), Xenopus TRα type a (TRAXA), Xenopus TRα type b (TRAXB; these two versions are due to the tetraploidization of the Xenopus genome) and to the sequence of Ho Huynh et al. (1996) (HO HUYHN). Obviously this last sequence is identical to Xenopus TRα type a.*

Genbank. A list of all Genbank codes for nuclear receptors can be found in Gronemeyer and Laudet (1995). The so-called *"Proteus anguinus "* sequence originates from Ho Hyuhn *et al.* (1996). TR sequences from *Ambystoma tigrinum* and *Necturus maculosus* were identified in Safi *et al.* (1997).

Mitochondrial 12S rRNA sequences are from Hedges and Maxson (1993) except for *Necturus maculosus* and *Proteus anguinus* sequences (Safi *et al.*, 1997).

Tree reconstruction

Phylogenetical reconstructions were performed using the MUST package (Philippe, 1993). When applied (Fig. 1B and 1C) 1000 bootstrap replicates were performed in order to test the robustness of the branches. Only branches with values above 60 can be considered as valid. For 12S rRNA sequences only transversions were used to calculate the tree since the transitions are saturated.

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