0013-7227/04/$15.00/0
Printed in U.S.A.

Endocrinology 145(2):760–772

doi: 10.1210/en.2003-0913

The Axolotl (*Ambystoma mexicanum*), a Neotenic Amphibian, Expresses Functional Thyroid Hormone Receptors

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Neotenic amphibians such as the axolotl (*Ambystoma mexicanum*) are often unable to undergo metamorphosis under natural conditions. It is thought that neoteny represents a deviation from the standard course of amphibian ontogeny, affecting the thyroid axis at different levels from the central nervous system to peripheral organs. Thyroid hormone receptors (TRs) that bind the thyroid hormone (TH) T_3 have been described in axolotl. However, the full sequences of TR were needed to better characterize the TH response and to be able to assess their functional capacity at the molecular level. We report that each of the α and β axolotl TRs bind both DNA and TH, and they activate transcription in response to TH in a mammalian cell-based transient transfection assay. Moreover, both TRs are expressed in axolotl tissues. Interestingly, each *TR* gene generates alternatively spliced isoforms, harboring partial or total deletions of the ligand-binding domain, which are expressed *in vivo*. Further, we found that in the axolotl, TH regulates the expression of *stromelysin 3* and *collagenase 3*, which are TH target genes in *Xenopus*. Taken together, these results suggest that axolotl TRs are functional and that the molecular basis of neoteny in the axolotl is not linked to a major defect in TH response in peripheral tissues. (*Endocrinology* 145: 760–772, 2004)

AMPHIBIAN METAMORPHOSIS is a spectacular postembryonic developmental process that is governed by thyroid hormones (TH). In the classical model *Xenopus laevis* as well as in other species, a peak of the circulating concentration of TH coincides with the climax of metamorphosis (1; reviewed in Refs. 2 and 3). Furthermore, the administration of TH in anuran tadpoles, even before the normal period of metamorphosis, triggers this process (4). On the contrary, blockage of endogenous TH production by goitrogens such as propylthiouracil or potassium perchlorate blocks metamorphic induction and produces giant tadpoles.

There is, however, a wide variety in the extent and importance of metamorphosis across different amphibian classes. In both urodeles and anourans, alternative strategies, such as direct development and neoteny, can be found (reviewed in Refs. 5–7). Neoteny, the ability to reproduce while conserving larval characters, can be either facultative, such as in many species of the genus *Ambystoma,* or obligatory, such as in *Necturus*, *Proteus*, or *Siren*. Salamanders of the genus *Ambystoma*, one of which is the axolotl, *Ambystoma mexicanum*, are a complex monophyletic group that lives in North America from northern Mexico to southern Canada (8, 9). Natural populations of *Ambystoma* can be purely neotenic, transforming, or polymorphic (*i.e.* some specimens undergo metamorphosis, whereas others reproduce as larvae), and it has been suggested that this represents an ecological adaptation useful for a better exploitation of the available resources (5). Phylogenetical analyses have shown that metamorphic failure evolved independently several times during *Ambystoma* evolution, suggesting that there is an increased ability to select this life history trait in all species of this genus (8). In addition, the phylogenetic data suggest that different defects may be responsible for the appearance of metamorphic failure in different *Ambystoma* species. In the laboratory, most *Ambystoma* species are able to undergo metamorphosis when treated with TH, whereas the concentration of TH necessary to trigger metamorphosis is highly variable (10). The most widely studied model system for neoteny, the laboratory axolotl, is at least in part derived from wild *A. mexicanum* and *A. tigrinum*.

A number of studies have focused on the molecular basis of metamorphic failure in the axolotl. The fact that the tissues of axolotl can respond to TH suggests that metamorphic failure in this animal resides mainly in abnormal TH production and a low rate of secretion of TSH (11). Several researchers also suggested that in contrast to *Xenopus*, the axolotl pituitary responds only very weakly to TRH (12, 13). Although it is likely that metamorphic failure in the axolotl can be attributed in large part to the low circulating levels of

Abbreviations: DBD, DNA-binding domain; HRE, hormone response element; LBD, ligand-binding domain; RXR, retinoid X receptor; TH, thyroid hormone; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

TH, hormone deficiency may not be the only factor involved. This is why other researchers used biochemical methods to assess the presence of TH receptors (TRs) as well as deiodinase activity in axolotl tissues (14). Further, using heterologous probes it has been shown that the *TRβ* gene can be up-regulated by TH treatment in axolotl (15). However, the full sequences of axolotl TRs, their expression levels, their response to TH, and their functional capacity have yet to be analyzed.

Most, if not all, of the biological effects of TH, *i.e.* the precursor, T_4 (L-T_4 or 3,3′,5,5′-tetraiodo-L-thyronine), and the more active compound, T_3 (3,3′,5-triiodo-L-thyronine), are mediated through two TRs, TR*α* and TR*β* (NR1A1 and NR1A2, respectively, in the nomenclature for nuclear receptors) (16–18). TRs are members of a large superfamily of nuclear receptors that act as ligand-dependent transcription factors (reviewed in Ref. 19). This family also includes vitamin D_3, steroid, and retinoid receptors. Nuclear receptors share conserved domains that are critical for their function: a short DNA-binding domain encompassing two zinc finger motifs (DBD) and a C-terminal hydrophobic ligand-binding domain (LBD) (19). The receptors bind specific DNA sequences termed hormone response elements (HREs), that contain repeats of a consensus half-site sequence (5′-AGGTCA-3′). TRs, like many members of this superfamily, form heterodimers with retinoid X receptor (RXR; NR2B), a receptor specific for 9-*cis*-retinoic acid, and bind to TH response element (TRE). These sequences usually consist of two half-sites arranged as a direct repeat separated by four nucleotides (DR4) (20), but sometimes may also include a palindromic element (HREpal) (21; for a review, see Ref. 22). In the absence of hormone, it has been shown that the RXR/TR heterodimer binds to corepressors to inhibit transcription (23, 24). In the presence of TH, the receptor undergoes a conformational change that induces the release of the corepressors and the recruitment of coactivators (reviewed in Ref. 25). This change results in a potent activation of target gene transcription.

To increase our knowledge of TR function in axolotl, we decided to study the TH response and *TR* genes of the laboratory axolotl in greater detail. We had previously cloned PCR products corresponding to partial sequences of two *TR* genes from the axolotl. Here, we obtained full-length clones and show that these two *TR* genes encode functional proteins that are able to bind DNA and TH like their *Xenopus* homologs. We also show that when transiently transfected in mammalian cells, these receptors are able to activate transcription in the presence of TH. Interestingly, we found that several isoforms of both TR*α* and TR*β* are likely to be produced by alternative splicing. Finally, we found that the *TRα* and *TRβ* genes are expressed in both larval and adult axolotl and that the genes encoding stromelysin *3* and collagenase 3, known to be TH responsive in *Xenopus*, are also regulated by TH in axolotl.

Materials and Methods

Animals

The laboratory has the official approval of the Centre National de la Recherche Scientifique for axolotl care, and all experiments were performed according to their guidelines. Laboratory axolotl were maintained in 10-liter tanks containing dechlorinated and filtered water at 18–20 C. Water was changed daily, and the animals were fed worms. The animals were treated with 5×10^{-8} M T_3 for 2, 5, or 10 d, and the water with hormone was changed daily. Four or five control animals and eight or nine treated animals were used at each point. Animals were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich Corp., St. Louis, MO), which was added to the water, and were subsequently killed by decapitation.

Isolation of axolotl TR cDNAs

We previously isolated RT-PCR fragments from axolotl corresponding to the central region of the *TRα* and *TRβ* genes (26). The fragments were used to screen a cDNA library constructed in λZAP GT11 from axolotl blastoma cells, a gift from Carl Seguin. Three clones were isolated for each gene of 4×10^6 plaque-forming units. Hybridization was performed overnight at 42 C in 50% formamide, 5× standard saline citrate, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 0.1 mg/ml denatured salmon sperm DNA. Membranes were washed twice in 2× standard saline citrate/0.1% sodium dodecyl sulfate at 37 C for 15 min.

Each cDNA was sequenced with a 373A ABI automated DNA sequencer (PE Applied Biosystems, Foster City, CA) with the PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit (PerkinElmer, Norwalk, CT). The three clones for both *TRs* were shown to be identical by restriction digestion and partial sequencing. The sequence of each cDNA was compared with the sequence of the RT-PCR fragments to ensure that we obtained full-length clones for both axolotl *TR* genes. Each sequence was subsequently compared with reference sequences using the FASTA program.

The accession numbers of the axolotl TRs are as follows: TR*α*,: AY174871; TR*β*, AY174872; TR*α* small isoform, AY280713; and TR*β* small isoform, AY280714.

In vitro translation and T_3 binding assays

The TRs and RXR, cloned in pSG5 (Stratagene, La Jolla, CA), were transcribed and translated using the TNT T7-coupled reticulocyte lysate system (Promega Corp., Madison, WI) according to the manufacturer's instructions in the presence or absence of [^{35}S]methionine (1000 Ci/mmol; Amersham Pharmacia Biotech, Arlington Heights, IL). The ^{35}S-labeled TRs were analyzed by SDS-PAGE in 8–18% gradient gels, followed by autoradiography. The level of protein-incorporated [^{35}S]methionine was measured using the trichloroacetic acid precipitation method according to the recommended protocol (Promega Corp.). An estimate neosynthesized [^{35}S]TR was calculated from [^{35}S]Met incorporation taking into account the presence of methionine within the rabbit reticulocyte lysate (5 μM[SCAP], Promega's technical recommendation). The T_3 binding affinity of the receptors was determined comparatively to that of human TR*β*1 in saturation experiments using [^{125}I]T_3 (3 μCi/μg; Amersham Pharmacia Biotech). *In vitro* translated receptors (1.5–3 μl) were incubated for 3 h at 20 C in 200 μl binding buffer [20 mM Tris-HCl, 1 mM $MgCl_2$, 2 mM EDTA, 100 mM KCl, 5 mM dithiothreitol, and 10% (vol/vol) glycerol, pH 7.95] containing 0.01–0.5 nM [^{125}I]T_3. Nonspecifically bound T_3 was always determined in parallel incubation with 0.1 μM unlabeled T_3 and subtracted from total bound T_3. Scatchard analyses of the binding data were performed as previously described (27).

Electrophoretic mobility shift assays

Full-length axolotl TR*α* and TR*β* as well as *Xenopus* TRs and *Xenopus* RXR*α* proteins were translated *in vitro* using the TNT kit (Promega Corp.). A DR4 probe (xDR4) from the *Xenopus TRβ* promoter encompassing the transcription start site (positions −12 to 18) (27) was labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Binding reactions were performed for 10 min using the radiolabeled DNA probe (1 ng, 40,000 cpm) and wheat-germ lysate in 10% glycerol, 10 mM HEPES, 30 mM KCl, 4 mM spermidine, 0.1 mM EDTA, 0.25 mM dithiothreitol, 1 mM Na_2PO_4, and poly(dI-dC) (1.5 μg). Unlabeled competitor oligonucleotides were included at the indicated molar excess in the binding reactions. Samples were loaded on a 5% nondenaturing polyacrylamide gel and electrophoresed for 2 h at 180 V.

Transient transfection assays

The axolotl *TRα* and *TRβ* cDNAs were subcloned into *Bam*HI/*Xho*I sites from a modified pSG5 vector. Ros 17/2.8 (rat osteosarcoma) or HepG2 (human hepatoma) cells were plated in DMEM (Bio Media, Paris, France) supplemented with 10% fetal calf serum at a density of 10^5 cells/dish. Transient transfections were performed using ExGen (Euromedex, Souffelweiersheim, France) with DNA mixture containing 1 μg total DNA, including 0.5 μg luciferase reporter construct (DR4-pGL2, Promega), 30 ng normalized vector, 0.1 μg receptor expression plasmid, and 0.37 μg pBSKSII (Stratagene, La Jolla, VA). Cytomegalovirus β-galactosidase (Stratagene), containing the bacterial *lacZ* gene expressed from the simian virus 40 early promoter, was also introduced into the cells and used as an internal control to account for transfection efficiency. Cells were washed after 6 h, and fresh medium was added together with 10^{-12}–10^{-6} M T_3. Cells were lysed 48 h after transfection and assayed for luciferase and β-galactosidase activities. Luciferase activity was divided by β-galactosidase activity to normalize for transfection efficiency. Transfections were performed in triplicate.

RT-PCR isolation in axolotl of homologs of Xenopus TR target genes

Given the nearly ubiquitous expression pattern of the *TRs* in vertebrates (28, 29), we used total RNA extracted from various axolotl adult tissues (muscle, gills, liver, *etc.*) and from TH-treated as well as untreated larvae. RNA was extracted from frozen tissues using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). The RNA samples were treated with RQ1 deoxyribonuclease [Promega; 2 U for 5–50 μg RNA in 10 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$, 37 C, 30 min], extracted using phenol/chloroform/isoamylic alcohol (25:24:1) and chloroform/isoamylic alcohol (24:1), and finally precipitated with ethanol.

One to 5 μg total RNA were reverse transcribed using random primers or specific primers and Moloney murine leukemia virus reverse transcriptase in 20 μl reaction mixture according to the manufacturer's instructions (Life Technologies, Inc., Moloney murine leukemia virus reverse transcriptase kit). The resulting cDNA was amplified by PCR in a 50-μl volume with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (PerkinElmer), 0.25 mM of each deoxy-XTP, 2.5 U *Taq* Gold DNA polymerase (PerkinElmer), and 300 ng of each primer.

Degenerate primers were designed using an alignment of published *Xenopus* nucleotide sequences and homologs from other vertebrate species according to the method described previously (30). The primers are described in Table 1. Most of these primers are degenerated and were used in a "touch-down" PCR assay (31) during which the hybridization temperature is reduced by 5 C every five cycles to allow hybridization of at least one of the primers of the degenerated mix. Denaturation and elongation temperatures remain unchanged. The complete PCR cycle is 94 C for 1 min, hybridization from 53–37 C for 1 min, and 72 C for 1 min for 40 cycles. To avoid contamination, all of the RT-PCR reactions as well as RNA extraction were conducted in a special nucleic acid-free room under positive air pressure using specific reagents (32, 33). For each pair of primers used, we prepared a control reaction without DNA to ensure that the PCR reactions were not contaminated by any mammalian genes routinely used in our laboratory. PCR products were cloned into the PCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Each plasmid was sequenced with a 373A ABI automated DNA sequencer with the PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit (PerkinElmer). Each amplified sequence was analyzed phylogenetically to verify that the sequence was from axolotl and did not result from a *Xenopus* contamination. The accession numbers of the axolotl genes are as follows: collagenase 3, AY139972; fibronectin, AY139973; and TIF2, AY139974

Semiquantitative RT-PCR assay

To study the expression of the various isoforms of axolotl *TRα* and *TRβ* and of the homologs of TH target genes, we designed specific primers (Table 1). Other primers were used as a positive control for the PCR reactions. In parallel to each PCR, a PCR reaction was performed to amplify the 28S rRNA to normalize the quantity of cDNA used for each sample. RTs were carried out with a pool of RNA for each point using the same amount of RNA for each animal. We previously determined the number of cycles to use to remain in the linear range. We used a standard PCR protocol consisting of 30 cycles for *TRα*, TRβ, and *collagenase 3*; 25 cycles for *stromelysin 3*, *fibronectin*, and *TIF2*; and 18 cycles for *28S* (denaturation at 94 C for 45 sec; hybridization at 55 C for 45 sec; elongation at 72 C for 45 sec). PCR products were separated on agarose gels and stained with ethidium bromide. The gels were then transferred to a Hybond N nylon membrane (Amersham Pharmacia Biotech) overnight in a solution of 0.5 M NaOH, and 1.5 M NaCl. DNA was fixed on the membrane by UV exposure (0.360 J/cm^2). Oligonucleotides specific for each PCR fragment were labeled by [γ-^{32}P]ATP using T4 polynucleotide kinase (Life Technologies, Inc.) and the conditions and buffers recommended by the enzyme supplier. The labeled oligonucleotides were purified on microspin G-50 columns (Amersham Pharmacia Biotech). After prehybridization (1 h at 37 C) with prehybridization buffer, hybridization was carried out overnight at 37 C with 500 ng labeled oligonucleotide. Subsequently, the membranes were rinsed several times for 10 min at 37 C and exposed with a phosphorimager film for about 12 h. The pixel quantification of each band was performed using IQMac version 1.2 software from Molecular Dynamics (Sunnyvale, CA).

Results

Isolation of full-length cDNAs encoding axolotl TRs

A cDNA library derived from axolotl limb blastoma cells was screened with the previously described RT-PCR fragments corresponding to *A. mexicanum TRα* and *TRβ* (26). For both the *TRα* and *TRβ* probes, we isolated cDNAs that were subsequently sequenced. We found that the sequences of these cDNAs were 100% identical with the sequences of the RT-PCR fragments that were used as probes during the screening procedure, suggesting that we isolated *bona fide* axolotl *TRs*. The two cDNAs encoding full-size TRα and TRβ proteins are, respectively, 1315 and 1428 bp long and contain in-frame start codons (at position 89 for the *TRα* cDNA and 307 for the *TRβ* cDNA) and stop codons (at position 1313 for the *TRα* cDNA and 1426 for the *TRβ* cDNA), resulting in proteins with lengths of 408 and 373 amino acids, respectively. The two cDNAs encode full-size *TRα* and *TRβ* gene products because they both contain in-frame stop codons 5′ to the first methionine.

The sequences of these clones were aligned with known *TRs* from vertebrates (Fig. 1). As expected, the axolotl TR sequences show strong sequence identities with known TRα and TRβ proteins. Overall, the axolotl TRα exhibits 86.8% and 88.8% amino acid identities with human and *Xenopus* TRα, respectively, whereas axolotl TRβ is 75.7% and 91.4% identical to the human and *Xenopus* TRβ. The difference in amino acid identity between axolotl TRβ and the human sequence is largely due to the divergence of the human TRβ in the A/B region. When this region is excluded, identity levels are 93.4% with the human sequence and 91.5% with the *Xenopus* protein. When specific domains are considered, we observed very strong conservation in the C domain (93–100% between axolotl TRα and its vertebrate homologs, 93–98% for TRβ) and in the E domain (88–90% between axolotl TRα and its vertebrate homologs, 92–93% for TRβ). Note that in each clone both the P box, which directly contacts the DNA, and the D box, which is another important feature of the DBD, are highly conserved. Among the 3 α-helixes that were found in the 3-dimensional structure of the DBD, helix H1, which contains the P box and establishes direct base-specific contact with the hormone response element, is fully conserved. In contrast, helix H2 and H3 in the axolotl TRβ contain some

TABLE 1. Oligonucleotides used in this study

Degenerated oligonucleotides used for touch-down RT-PCR	
Fibronectin	
FIBRO51 (5′1)	ACTG(A/G)CATTCACTGATGTGG
FIBRO52 (5′2)	ACTC(A/G)AG(C/T)CCTGAGGATGGA
FIBRO31 (3′1)	AGGAA(A/C)CGCAGGTTGGATGG
FIBRO32 (3′2)	GGIGAGCT(C/A)CG(A/G)GCATTGTC
Collagenase 3	
COLLA51 (5′1)	TT(C/T)TT(C/T)G(A/G)(C/T)(C/T)T(A/G)GA(A/G)GTGAC
COLLA52 (5′2)	(A/C)G(A/G)TG(T/C)GGIGTICC(C/T)GA(C/T)GT
COLLA31 (3′1)	GG(A/G)T(A/G)(T/C)TC(A/G)TA(T/G)GC(A/G)GCATC
COLLA32 (3′2)	CGCCAGAAGAA(T/C)CTGTC(C/T)TT
TIF2	
TIF51 (5′1)	CGIGA(A/G)CA(G/A)GA(G/A)A(G/A)(C/T)AA(G/A)TA
TIF52 (5′2)	A(A/G)(C/T)(G/T)T(C/A)AA(G/A)CCIGA(T/C)AATG
TIF31 (3′1)	GAIAI(G/A)G(C/T)(A/G)AA(G/A)CACTGCAT
TIF32 (3′2)	GC(A/G)(G/C)(C/T)(C/A)A(C/G)I(G/A)(G/T)ICC(A/G)TC
TIF33 (3′3)	AG(C/T)(C/T)T(G/A)C(T/A)(T/C)TTIGTTTGIGC
Specific oligonucleotides used for semiquantitative RT-PCR	
28S	
28S5 (5′)	CAGGTGTCCTAAGGCGAGCT
28S3 (3′)	TATTAGTGGGTGAACAATCC
28SP (probe)	AGCGTTCATAGCGACGTCGC
Stromelysin 3	
ST5 (5′)	TTCCCTGCTCAGCCAGACAG
ST3 (3′)	TGGGTCTTAGGAAAGAAGGC
STP (probe)	AAAGTGAGGCGCACGATCGC
Collagenase 3	
COLLA5 (5′)	ATGTGGGGGAATATAACGTG
COLLA3 (3′)	CCAATTCTTTTACCAGGGGG
COLLAP (probe)	TCCCCAGAAATCTGAAATGG
Fibronectin	
FIBRO5 (5′)	CCAAAAGGGGATGATGACAG
FIBRO3 (3′)	CCGAGGTGCTGTCTGGTGAC
FIBROP (probe)	CAACTGCACGGCCTCCGACC
TIF2	
TIF5 (5′)	TTCTGATGCGTTTCCTGGTT
TIF3 (3′)	CTGGAAGGGAATGTTGTATT
TIFP (probe)	CGCCATTCACTAAAGACTTT
TRα	
α5 (5′)	AATATGACGGCTGTTGCATT
α3 (3′)	CCACCCTCGTGATGGCCGGG
ΔA3 (3′)	CTGATCTTCACAAGGCAGGCAGG
αP (probe)	GCGGCGGCGCAAAGAGGAAA
TRβ	
β5 (5′)	GTCACCGACGCACACATGGC
β3 (3′)	TTTCCCCATTCAGCGTTAAG
ΔB5 (5′)	TTGGCATGGCCACAGATTCCTGA
βP (probe)	ATGCCCCAGATGGTGGGAAA

The *upper part* of the table shows the oligonucleotides used in touch-down PCR for isolation of new axolotl sequences, whereas the *lower part* presents oligonucleotides used in semiquantitative RT-PCR assays. In each case the oligonucleotide used as a probe is indicated.

substitutions compared with either the human or *Xenopus* sequences. These domains are important for protein-protein interactions and, in the case of H3, for heterodimerization with RXR and nonspecific contacts with DNA. In the LBD of both TRα and TRβ we also find very strong conservation in the 12 α-helixes that form the core of the 3-dimensional structure (34), suggesting that the TRs from axolotl have all the features to bind TH and to *trans*-activate target genes in response to T_3. The helixes H2, H3, H5–6, H9, H11, and H12 are very highly conserved. Interestingly, all of the amino acids that are in direct contact with the ligand in the 3-dimensional structure of the rat TRα LBD (namely N228, R277, R311, R315, and S326, which is an N for TRβ receptors; see Fig. 1) are fully conserved in the axolotl TRs, as are the regions containing the autonomous *trans*-activation domain AF2-AD. The A/B region is very poorly conserved when mammalian proteins are compared with those from axolotl, but a significant degree of sequence identity exists within this region between the amphibian TRα and TRβ proteins. In both *Xenopus* and axolotl, the A/B region of the TRβ protein is very short, suggesting that we have isolated a specific isoform of axolotl TRβ that probably corresponds to the *Xenopus* TRβ isoform, the expression of which is directly regulated by T_3 (35). Although the D domain contains more substitutions than the other domains, it is quite conserved between TRα and TRβ.

Functional characterization of the axolotl TRs

To test the abilities of these cDNAs to encode functional TRs, we carried out a series of *in vitro* assays. First, we used

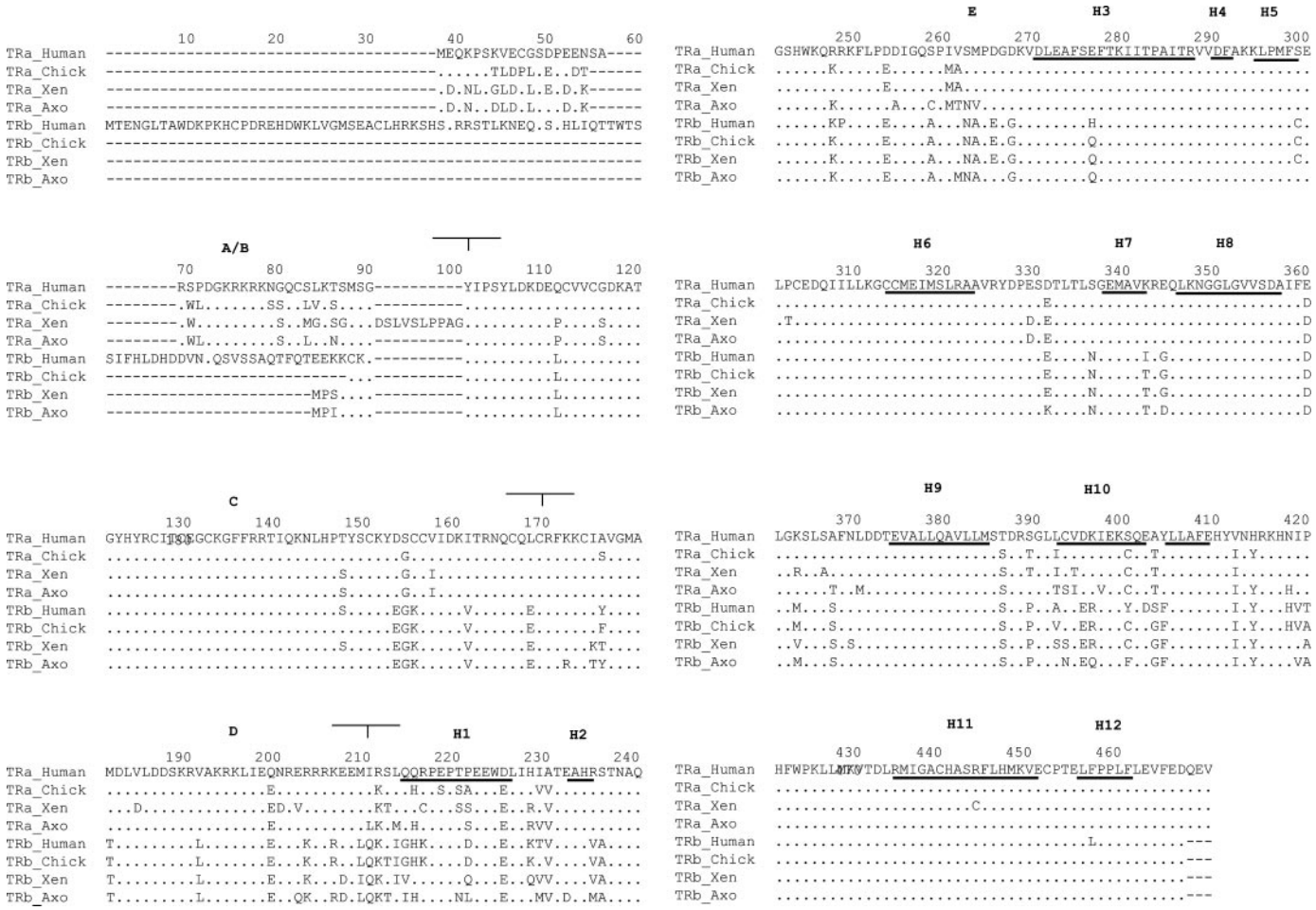

FIG. 1. Amino acid sequence alignment of *TRα* and *TRβ* from human, chicken, *Xenopus*, and axolotl. The various structural domains and their boundaries are indicated. For the LBD (also called the E domain) the 12 helixes found in the three-dimensional structure of the mammalian TRs are indicated. The accession numbers are as follow: human *TRα*, AAA66021; chicken *TRα*, CAA68792; *Xenopus TRα*, AAA49970; axolotl *TRα*, AY174871; human *TRβ,* CAA28412; chicken *TRβ*, CAA35544; Xenopus *TRβ*, AAA49657; and axolotl *TRβ*, AY174872.

electromobility shift assays to test whether the axolotl TRs synthesized *in vitro* bind to DNA. We used the DR4 element from *Xenopus* (xDR4) as the labeled probe and a number of other TREs as competitors. The xDR4 was isolated from the promoter of the *Xenopus TRβ* gene and has been characterized as the TRE mediating TRβ autoinduction during *Xenopus* metamorphosis (reviewed in Ref. 3) (Fig. 2A). As shown in Fig. 2B, neither axolotl TRα nor TRβ can bind to this element alone (lanes 2 and 11), but they form a strong heterodimer, which binds to the xDR4 probe in presence of the *Xenopus* RXRα protein (lanes 3 and 12). To ensure the specificity of the binding of the TR-RXR heterodimers to the xDR4 probe, we performed competition experiments with four TREs: the xDR4 element itself, a consensus DR4 sequence, a palindromic element (HREpal) (15) and an unrelated (NS) oligonucleotide (Fig. 2A). We also used more complex elements, such as the one upstream the myelin basic protein gene (data not shown). For both TRα and TRβ, the binding can be competed out by the xDR4 sequence (Fig. 2B, lanes 4, 5 and 13, 14), but not by an unrelated oligonucleotide (lanes 9 and 19). These competition experiments also reveal that axolotl TRα and TRβ proteins can bind to a perfect consensus DR4 element (lanes 6, 7 and 15, 16) as well as the HREpal (lanes 8, 9 and 17, 18). All of these results are consistent with observations made with *Xenopus* TRα and TRβ proteins in similar experiments (data not shown). Taken together, these data suggest that the DNA-binding characteristics of axolotl and *Xenopus* TRs are very similar, if not identical.

We next examined the *trans*-activating properties of the axolotl TRs using transient transfection assays. These assays were performed in osteosarcoma Ros 17.2/8 cells, because these cells contain a high level of RXR, which is sufficient to provide a strong heterodimerization partner for transfected TRs. In addition, these cells contain very low levels of TR that avoid interference between endogenous and transfected receptors (36). The results show that axolotl TRα and TRβ proteins are both able to activate transcription of the xDR4-luciferase reporter construct in a T_3-dependent manner (Fig. 3A). In addition, we performed dose-response experiments by transient transfection of HepG2 cells with the TR expression vectors and the xDR4 reporter construct. This experiment clearly shows that the transcriptional activity of the receptors can be detected at 0.1 nM and is maximal at 1 μM. All of the receptors activate transcription within the same

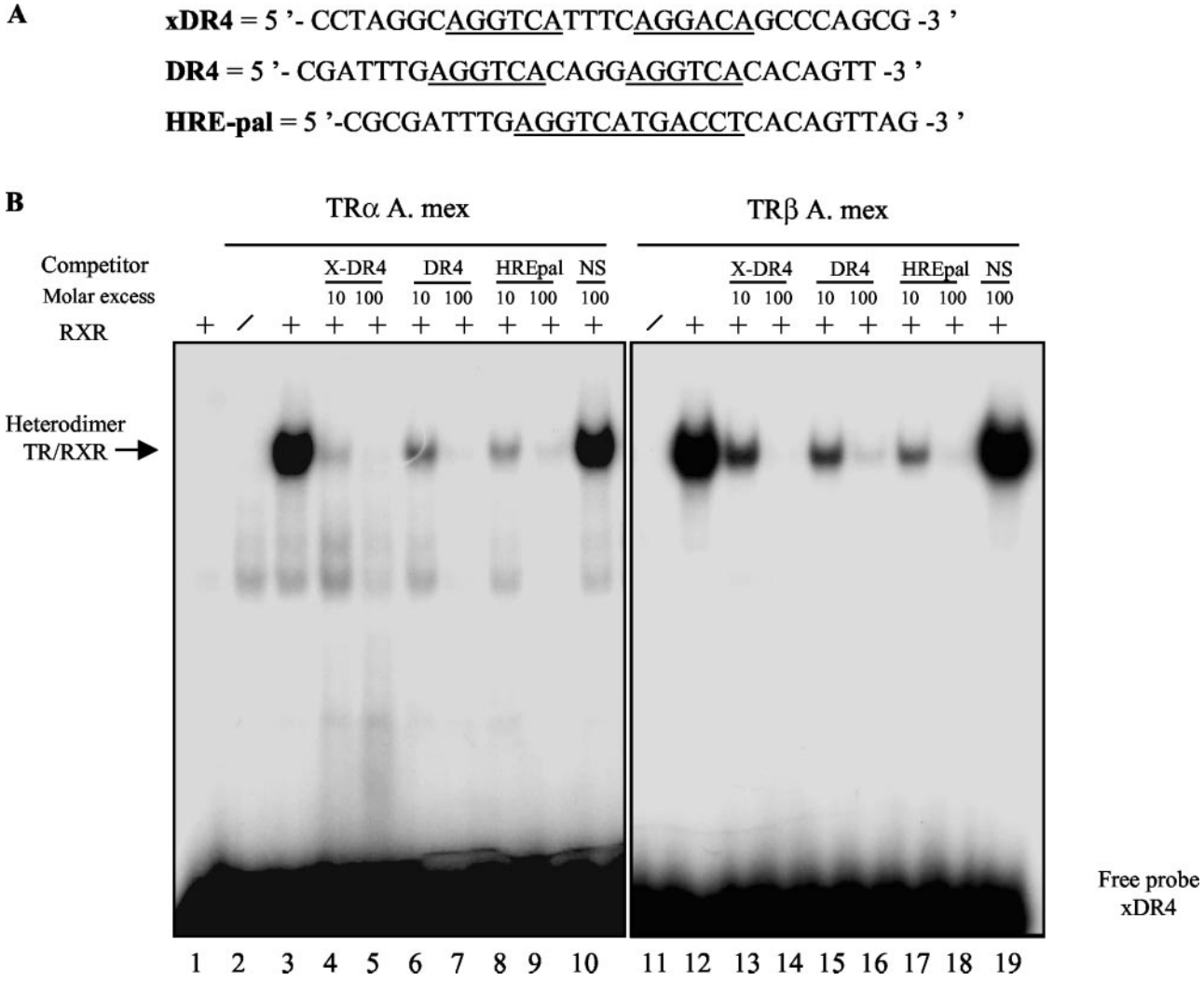

FIG. 2. A, Sequences of the probes used. Consensus half-site are *underlined*. B, EMSA experiments. The labeled probe is the DR4 of the *Xenopus TRβ* promoter (xDR4) in competition with the others at the indicated molar excess. The experiments were performed with *in vitro*-synthesized TRα (lanes 1–10) or TRβ (lanes 11–19). For each lane, *in vitro*-synthesized *Xenopus* RXRα was added to allow dimerization with axolotl TR, and the *arrow* shows the heterodimer RXR/TR binding the X-DR4-labeled probe. NS, Nonspecific probe. This experiment was performed three times with identical results.

range, except axolotl TRβ, which seems to be more potent than the others. This difference should nevertheless be interpreted with caution (see *Discussion*).

All of these data suggest that the axolotl TRs are, like their *Xenopus* homologs, able to bind to T_3. To directly test this hypothesis, we measured the binding affinity of the *in vitro* translated receptors to labeled T_3. Scatchard plots show that the binding affinity of the axolotl TRs is comparable to the affinity of *Xenopus* TRs to T_3 (K_d values for axolotl TRα, 60.6 pM; for axolotl TRβ, 15.1 pM; Fig. 4). All of these data suggest that axolotl TRs are functional because they can bind to DNA, heterodimerize with RXR, bind to T_3, and activate transcription in mammalian cells in response to this hormone.

Presence of different isoforms of TRα and TRβ

We studied the expression of *TRα* and *TRβ* genes by RT-PCR with 5′ and 3′ primers specific for the C and E domains, respectively. We noticed, in addition to the expected bands, shorter fragments for both *TRα* and *TRβ*. These bands were cloned and sequenced. For *TRα*, we found that the shorter band corresponds to a deletion of 147 nucleotides (data not shown). Strikingly, the boundaries of this deletion, which we call *TRΔα*, corresponds to the boundaries of the human *TRα* exon 7, suggesting that this exon may be left out in some mRNA transcripts (19) (see Fig. 5). In this case, the resulting protein contains an in-frame deletion of 49 amino acids that corresponds to the region of helixes H3 and H4 and is unlikely to bind to T_3 because this region encompasses some of the residues that are in direct contact with the ligand. For *TRβ*, the cloned, shorter RT-PCR fragment includes a deletion of 309 nucleotides, the boundaries of which coincide with the human *TRβ* exon 6. As for *TRα*, this strongly suggests that the shorter fragment corresponds to an alternatively spliced isoform of the axolotl *TRβ*, which we call *TRΔβ* (Fig. 5). Because the deletion of this exon in axolotl *TRβ* changes the reading frame of the resulting transcript, and a

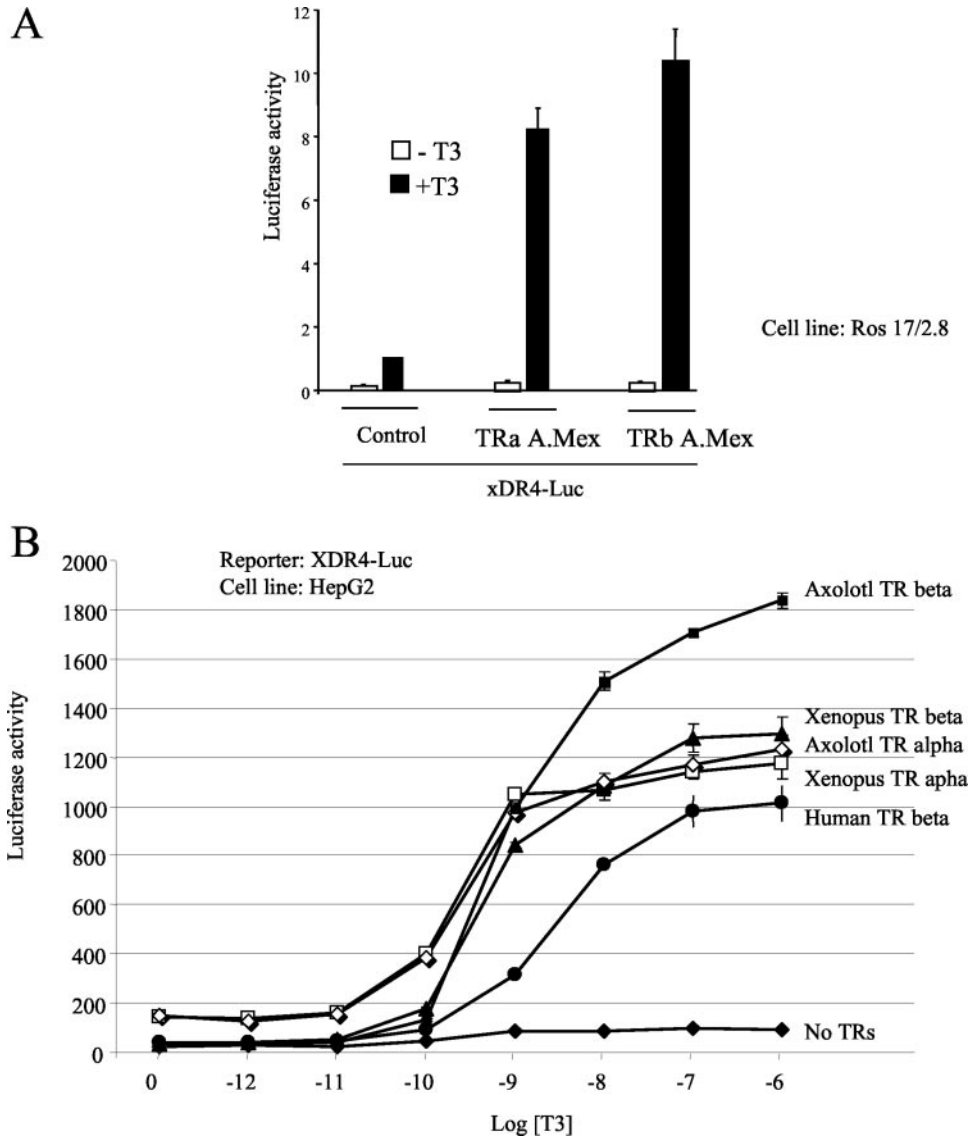

FIG. 3. A, Transcriptional activity of axolotl TR in response to T_3. The data are presented as luciferase activity for axolotl TRα and TRβ in response to 10^{-7} M T_3. The pSG5, pSG5-TRα, and pSG5-TRβ were transfected together with xDR4 LUC reporter construct in Ros 17.2/8 cells, and T_3 was added to the cell culture. B, Dose-response experiment performed by transient transfection of HepG2 cells. The luciferase activity was plotted against the log of the T_3 concentration. The activity of the axolotl TRs was compared with that of the *Xenopus* receptors as well as to human TRβ activity. The transfections were performed in triplicate, and the whole set of transfections was performed twice with identical results.

stop codon is present two amino acids downstream from the alternative spliced boundary, this alternatively spliced variant generates a truncated protein. This 90-amino acid-long product will contain only the A/B and C regions, and no D and E domains. This product will thus be unable to bind T_3 and is unlikely to bind to DNA because it lacks the helix H3 of the DBD, which is important for nonspecific interaction with the sugar-phosphate backbone of the DNA molecule.

To check that these isoforms do not represent RT-PCR artifacts, RNA from whole axolotl larvae were analyzed by RT-PCR with primers specific for each isoform (Fig. 5, A and D, and Table 1). *TR*α- and *TR*β-specific primers were used as positive controls. Interestingly, we observed a band at the expected size for full-size *TR*α and *TR*β transcripts. We also found strong expression of the *TR*$\Delta\alpha$ isoform, whereas *TR*$\Delta\beta$ was expressed at a much lower level. These results suggest that both isoforms are expressed and should correspond to *bona fide* transcripts, which may play a role in regulating the T_3 response during axolotl postembryonic development.

We then tested whether the expression of the *bona fide* TR transcripts as well as the transcripts encoding the isoforms was regulated by T_3 using a semiquantitative RT-PCR assay. Surprisingly, the results (data not shown) reveal no significant variation in *TR*α, *TR*β, *TR*$\Delta\alpha$, or *TR*$\Delta\beta$ expression under T_3 treatment in the head or the tail of the axolotl.

TH response in axolotl

As axolotl tissues expressed functional TR mRNAs, we next studied the influence of T_3 on the regulation of other genes that are known to be important in *Xenopus* metamorphosis. In *Xenopus*, it has been proposed that TH treatment induces the expression of early response genes, such as *TR*β itself, as well as transcription factors, such as *BTEB* or *TH-bZIP*, and matrix metalloproteases, such as *stromelysin 3* (37–39). It has also been proposed that *TR*β activates the transcription of late genes, such as *collagenase 3* and *fibronectin*. Using RT-PCR and degenerate primers, we thus cloned axolotl homologs of matrix metalloproteases (*stromelysin 3* and *collagenase 3*) and other matrix-associated genes, such as *fi-*

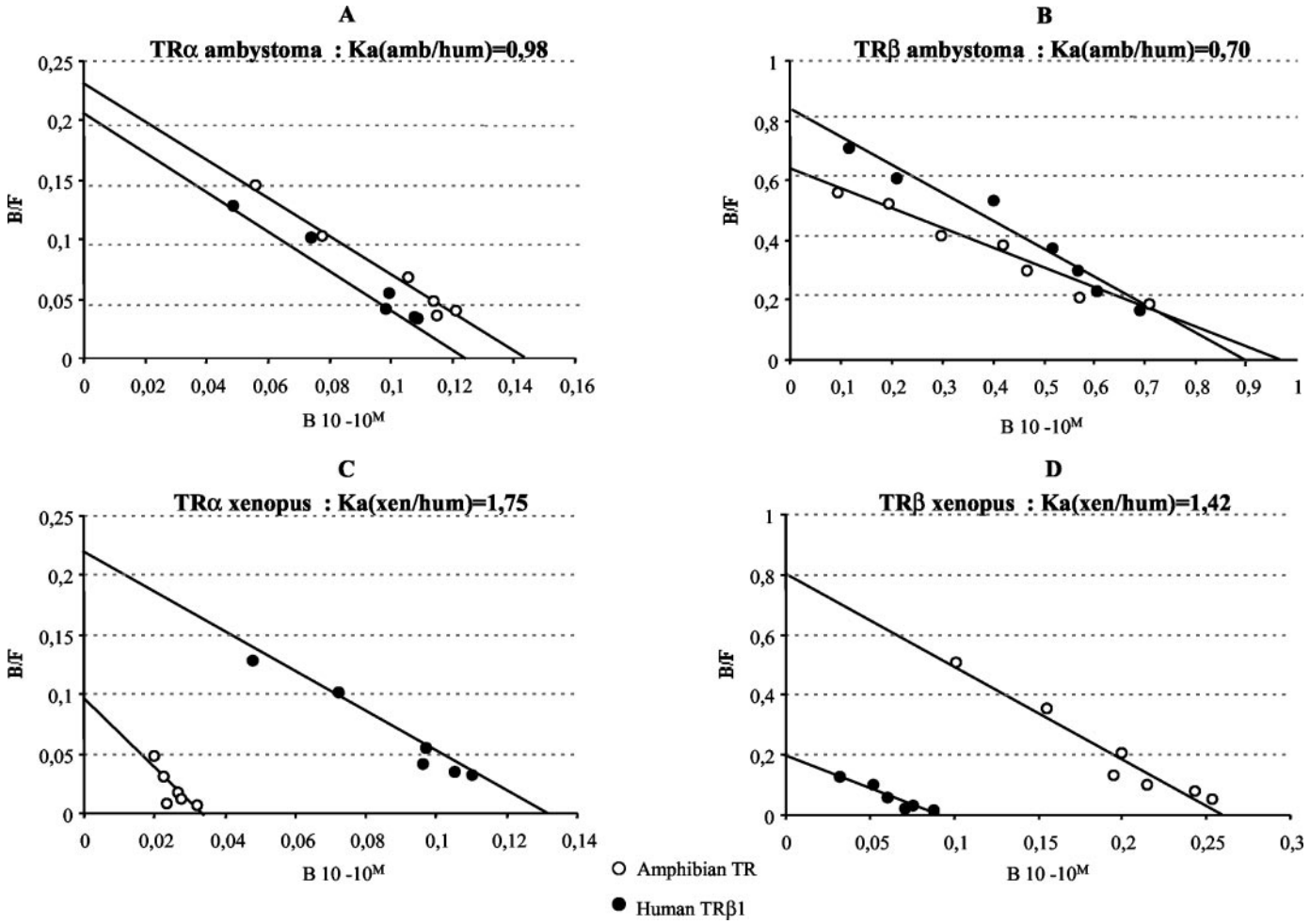

FIG. 4. T_3 binding of axolotl TRs compared with their *Xenopus* homologs. Each Scatchard plot of a representative experiment is presented with the data obtained using human TRβ1 as a control. In each case the K_a value is compared with that for human TRβ1. ○ and ●, Amphibian and human data, respectively. B, Bound T_3; B/F, bound to free T_3 ratio. Each experiment was performed at least four times.

bronectin. In addition, we isolated an axolotl homolog of the transcriptional coactivator *TIF2*, which plays an important role in mediating TR transcriptional activity in mammals (see Ref. 19 for references). The RT-PCR fragments were cloned and sequenced, and their authenticity was assessed by careful phylogenetic analyses. We found that the position of each of the sequences in the tree is consistent with the phylogenetic position of its respective species of origin (data not shown). Using these sequences we designed specific primers (Table 1) that are able to amplify the axolotl sequence in semiquantitative RT-PCR experiments.

Using samples of head and tail tissues of T_3-treated animals, we observed that the expression of both *stromelysin 3* and *collagenase 3* is induced by T_3 treatment in the tail and the head (Fig. 6). Interestingly, the time course of activation of these two genes varies according to the region of the animal used. In the head, *stromelysin3* is rapidly induced 2 d after T_3 treatment, whereas *collagenase 3* induction requires a longer exposure (strong activation after 10 d). In contrast, in the tail, both genes are rapidly induced. *Fibronectin* and *TIF2* expression were not affected by T_3 treatment (not shown). All of these data suggest a complex response mechanism induced by T_3 treatment in axolotl larvae. This concept is consistent with our functional results showing that axolotl TRs are able to activate transcription in response to T_3 in transient transfection assays.

Discussion

Axolotl TRs are functional

Our analysis clearly shows, in accordance with previous studies (14), that TRs are present, functional, and expressed in axolotl tissues. It was important to characterize in more detail axolotl TRs to know whether their ability to respond to TH is comparable to that of *Xenopus* TRs. At the level of sequence, the extent of conservation between *Xenopus* and axolotl TR proteins is impressive. The DNA-binding C domains of *Xenopus* and axolotl TRα are 100% identical, whereas for TRβ only three substitutions are obvious in helix 2, which plays no direct role in DNA binding or heterodimerization. Helix 3, which contains the T and A boxes important for DNA binding affinity and response element selection, is also well conserved. The few sequence differences in this helix between axolotl and *Xenopus* TRs are conservative substitutions (*e.g.* Q to E at position 192, or K to R at position 196), which do not alter the potential to form an helix. In the LBD,

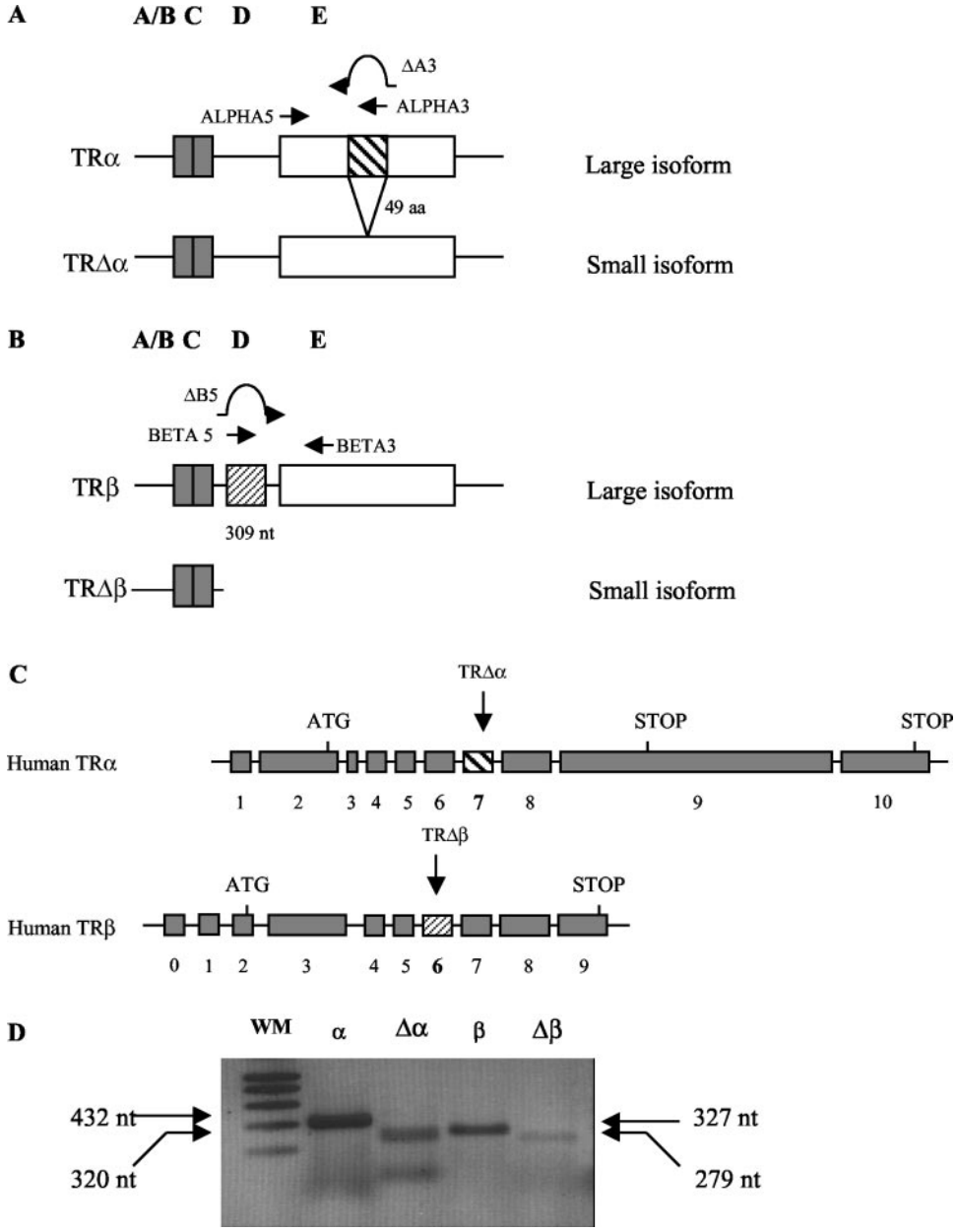

FIG. 5. A, Structure of the axolotl *TRΔα* isoform. The deletion of 49 amino acids removes part of the LBD. B, Structure of the axolotl *TRΔβ* isoforms. The deletion of 309 nucleotides results in a change in the reading frame of the transcript. Thus, this isoform does not contain a LBD. For A and B, the primers used to test the expressions of the different isoforms are indicated by *arrows*. C, Genomic organization of the human *TRα* and *TRβ* genes. The axolotl *TRΔα* isoform results from a deletion of an exon homologous to exon 7 in the human *TRα* gene. The axolotl *TRΔβ* isoform corresponds to the deletion of an exon homologous to exon 6 of the human *TRβ* gene. The functional domains are indicated *above* the genomic structure. Exons are represented in proportion to their size in base pairs, whereas intron lengths are arbitrary. D, Detection of the *TRΔ* isoforms in axolotl tissues. RT-PCR amplification of all the *TR* isoforms with cDNA coming from whole axolotl larvae. WM, Molecular weight marker. The sizes of the expected PCR products are indicated. The experiments were repeated twice with similar results.

several lines of evidence also suggest that this domain is functional: 1) all of the amino acids that are in direct contact with the ligand in the three-dimensional structure are conserved; 2) none of the mutations that are known to occur in the human *TRβ* gene in TH resistance syndromes can be found in the axolotl TRs (40); 3) all regions known to be important for the various functions of these receptors are well conserved (helix 9 for dimerization, helixes 11 and 12 for *trans*-activation function, and helixes 3 and 4 for coactivator binding) (see Ref. 19 for references); and 4) all of the substitutions observed in the axolotl genes are in loops and not in helixes, suggesting that substitutions accumulate in nonessential regions. Thus we found no substitutions suggesting any important functional differences between *Xenopus* and axolotl TRs.

The functional characterization of the axolotl TRs clearly shows that they bind DNA and heterodimerize with *Xenopus* RXR in a manner indistinguishable from the *Xenopus* TR orthologs. We found that TR*α* and TR*β* are able to bind to a natural response element, namely the DR4 element found upstream of the *Xenopus TRβ* gene. In addition, using competition experiments, we found that axolotl TRs can recognize palindromic elements, suggesting that in axolotl, as in *Xenopus*, TRs can bind to a wide variety of response elements. The *trans*-activation assays that we performed in mammalian cells also suggest that the axolotl TRs are functional because we observed a very strong rise in transcriptional activity of the reporter gene in response to TH. The conditions we used resulted in very low activity of the reporter gene in the absence of exogenously transfected TRs and did not allow us to test whether the axolotl TR exhibits repressive activity in the absence of TH, as has been reported for TRs from other

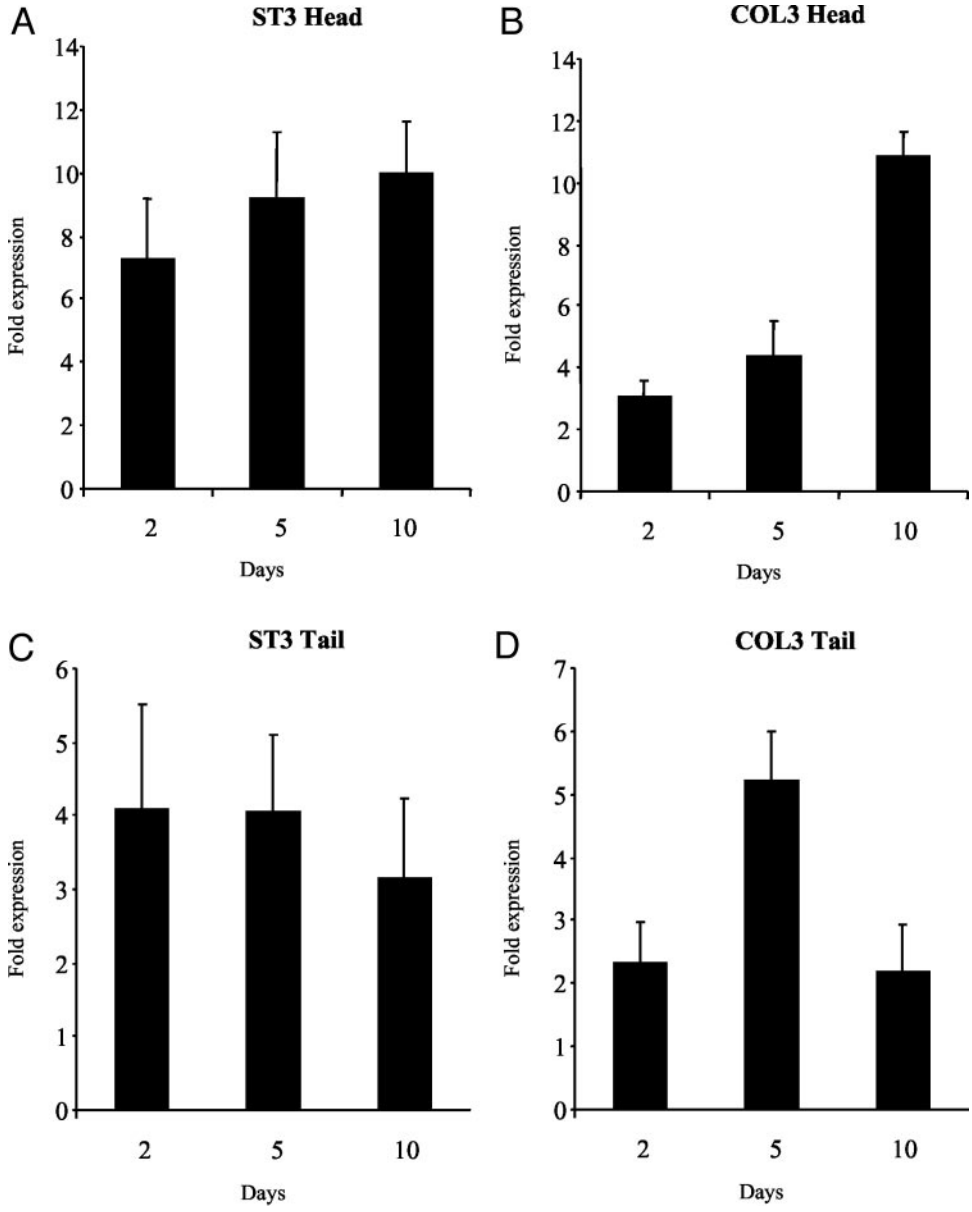

FIG. 6. Induction of target gene expression after T_3 treatment. A, Stromelysin 3 mRNA in the head. B, Collagenase 3 mRNA in the head. C, Stromelysin 3 mRNA in the tail. D, Expression of collagenase 3 mRNA in the tail. The histograms represent the average radioactivity quantified for each gene normalized with the 28S rRNA for each time of treatment as fold expression compared with the untreated animals. For each time point, RT-PCR was performed with a mixture of RNA from different animals (same amount for each animal). RT-PCR was performed twice independently. In the head, as in the tail, *stromelysin 3* and *collagenase 3* expression is induced by T_3.

species. The activation seen with the axolotl TRs in dose-response experiments is comparable to results obtained with the respective *Xenopus* receptors, except for TRβ, which appears to be a little more efficient in axolotl than in *Xenopus*. However, these data should be interpreted with caution. Indeed, the *trans*-activation experiments were performed in a heterologous cell system containing mammalian coactivators. The detailed comparison of the *trans*-activation potentials of *Xenopus* and axolotl TRs should be made in *Xenopus* and axolotl cells to avoid possible differences in affinity between the various coactivators and the TRs. Study of the direct binding of TH to the receptor suggests that, at least at the level of hormone-receptor interaction, *Xenopus* and axolotl TRs are extremely similar, as the affinities of the receptors for the hormone are very similar in both species.

All of these observations fit very well with the finding that TH induces metamorphosis in axolotl (Ref. 41 and references therein). The study of several target genes, such as *TRβ*, *collagenase 3*, *stromelysin 3*, and *fibronectin*, revealed that a clear TH response can be observed in axolotl, as observed in *Xenopus* (see below).

New isoforms of TRα and TRβ

We identified isoforms for both axolotl TRα and TRβ. For TRα, we found an in-frame deletion of the exon homologous to exon 7 of the human *TRα* gene, which results in a deletion of 49 amino acids localized in the LBD. Surprisingly, we isolated the same isoform in the newt *Pleurodeles waltii* (Safi, R., and V. Laudet, unpublished observations) as well as in a teleost fish, the tilapia (*Oreochromis niloticus*) (42). We thus think that this isoform corresponds to an alternatively spliced variant with a large deletion inside the LBD. This deletion corresponds to the region of helixes H3 and H4,

which are important for the ligand-binding pocket as well as for the interaction with coactivators. We have also functionally characterized this isoform and found that it exhibits a very weak DNA-binding ability on the XDR4 element (data not shown). We interpret this result as an inability of this protein to interact with RXR because the large deletion in the middle of the LBD disrupts the RXR heterodimerization interface that is present in the LBD, mainly in helix H9. Consistent with this idea, this isoform is transcriptionally silent and does not exhibit a clear dominant negative activity when tested in transient transfection assays (Safi, R., unpublished observations). As it is expressed *in vivo* and found in various unrelated animal species (amphibians and teleost fishes), we propose that it has a biological function that remains to be defined. For TRβ, we found another alternative splice variant resulting in a deletion of the homolog of exon 6, which generates a 90-amino acid product that is unable to bind both DNA and TH and is transcriptionally inactive *per se* and in a dominant negative activity assay (data not shown). This isoform is expressed *in vivo* at lower levels than the *TR*$\Delta\alpha$ isoform, and its expression is not changed during TH-induced metamorphosis.

Nearly all known *TR* genes generate isoforms that are unable to bind T_3 and are transcriptionally silent. This is the case for the TRα2 isoform in mammals, which is a C-terminal, alternatively spliced variant of the *bona fide* TRα1 and has a dominant negative activity in transient transfection assays (43–45). In addition, the human and mouse TRα genes encode isoforms generated from an internal promoter located in exon 7 that represent a truncated version of the LBD (46). Careful genetic dissection of the functions of the various isoforms encoded by the TRα locus have revealed that these short isoforms play an important role in buffering the actions of both TRα and TRβ (29, 47, 48). This is also the case in chickens, where several transcriptionally inactive, N terminally truncated, TR proteins are generated by alternative in-frame ATG usage (49). The physiological role of these TH-independent isoforms is still unclear. Perhaps it is physiologically relevant for animals to be able to generate different TR proteins, which are unable to bind T_3 and/or to activate target genes at certain times during embryogenesis or postembryonic development.

TH response in axolotl

In *Xenopus*, numerous TH-dependent genes were isolated from the resorbing tail using a PCR-based subtractive hybridization procedure (50). Half of the 17 up-regulated genes were shown to respond rapidly to and be directly regulated by the hormone, because their induction is not abrogated by cycloheximide treatment (51). Four of these early response genes are transcription factors (*TR*β, *xBTEB*, *TH/bZIP*, and *FRA-2*), which can, in turn, induce a cascade of TH-dependent gene transcription (39, 52). Among the other early-induced genes are the enzyme *type III deiodinase* and a matrix metalloprotease, *stromelysin 3* (52). Another class of genes, the delayed response genes, is induced about 24 h after TH treatment. These delayed response genes include extracellular matrix components, such as *fibronectin* and *integrin* α_1, and other proteolytic enzymes, such as *FAP*α, *peptidase E*, or *collagenase 3* (52). Study of the expression of these genes during metamorphosis has led to a model in which a unique hormone (TH) induces distinct, and even opposite, processes through independent regulatory programs (Fig. 7) (37, 38). It has been proposed that when TH surges at the beginning of metamorphosis, it binds to TRα, which is expressed early in postembryonic development and is ubiquitously expressed in tadpoles. Liganded TRα probably induces early genes such as *TR*β, which subsequently up-regulates its own transcription by an autoregulatory loop and thus mediates the response to TH. *TR*β as well as the other transcription factors subsequently induces different cascades of gene activation. By studying the *in situ* localization of the transcripts of all of these genes in the tail as well as the head of tadpoles Berry *et al.* (37, 38) suggested that the early response transcription factors *xBTEB*, *TH/bZIP*, and *FRA-2* induce the growth of adult tissues such as cartilage, nervous tissues, adult epidermis, and adult muscles (Fig. 7). In contrast, *TR*β, by regulating the delayed response genes such as *collagenase 3* and *fibronectin*, is implicated in the resorption of larval tissues such as cartilage, muscle, and epidermal lamellae in the tail. *Stromelysin 3*, which is also an early response gene, is implicated in the resorption of larval structures in the tail and other tissues.

We have isolated axolotl homologs of some of the genes implicated in the TH cascade in *Xenopus*, such as *TRs*, *collagenase 3*, *stromelysin 3*, and *fibronectin*. Despite extensive efforts, we failed to isolate homologs of the early transcription factor genes such as *BTEB*, *FRA-2*, or *TH/bZIP*. We found that only two of the axolotl genes isolated, *collagenase 3* and *stromelysin 3*, are regulated by TH, even if in some cases (*collagenase 3* in the head) this induction requires a relatively long delay. At present, the reason and significance of these differences between axolotl and *Xenopus* remain unclear, and more studies are needed to compare the genetic programs induced by TH in the two species. It is striking that we did not find any obvious induction of TR genes after TH treat-

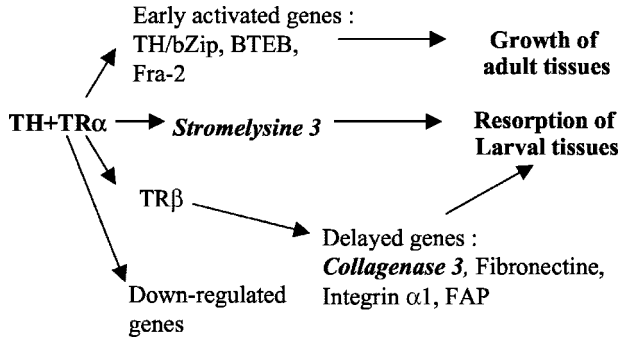

FIG. 7. Hypothesis for a gene expression regulation cascade during *X. laevis* metamorphosis. TH interacts with the receptor TRα, which activates the expression of the early genes, among them *TR*β. Subsequently, the hormone interacts with TRβ, activating the expression of the delayed genes that are responsible for the resorption of larval tissues. The transcription factors, except *TR*β, among the early induced genes play an important role in the growth of adult tissues. BTEB, Basic transcription element-binding protein; FAP, fibroblast-activated protein; Fra-2, Fos-related activator 2; TH/bZip, TH-activated protein with bZip domain. The *collagenase 3* and *stromelysin 3* genes that we found regulated by TH in axolotl in this study are *italicized*.

ment in either the tail or the head of axolotl. This result differs from those obtained by other researchers (53) and may be due to age- or tissue-specific factors, as gills were used by Yaoita and Brown (53a) to measure TR induction. A definitive answer to this question requires a more comprehensive analysis of TR expression *in vivo* in axolotl.

Interestingly, in the course of this study we observed in axolotl a strong TH-dependent up-regulation of *collagenase 3*. In *Xenopus*, *collagenase 3* is expressed in fibroblasts, which either line or surround the notochord or are located beneath the epidermal lamellae (11, 12). During tail resorption, these fibroblasts invade the neighboring tissues and are crucial in the tail resorption process. It is striking to find induction of *collagenase 3* in axolotls, because the tail of the axolotl, like that in other urodeles, does not regress during metamorphosis. These data suggest that the axolotl tail is TH responsive, and that *collagenase 3* induction itself is not sufficient to induce a tail resorption program. Alternatively, it is possible that in axolotl, a block of *collagenase 3* action occurs downstream of their activation by TH. In contrast, the fact that we found TH induction of *stromelysin 3* is consistent with previous observations in *Xenopus*, where *stromelysin 3* is induced in the dorsal and ventral fins. In axolotl, the first morphological event after TH treatment is the resorption of the ventral and dorsal fins. Thus, it is tempting to predict that *stromelysin 3* is induced in the fins of the axolotl. It will be interesting to determine in which cell types the induction of these various genes occurs and if other matrix metalloproteinases, such as gelatinase A or MT3-MMP, that have been described as being involved in tail resorption are also regulated (53).

Axolotl: a promising model for postembryonic development

Considerable interest is directed to understanding evolution of developmental processes and their link to morphological or life history traits. Axolotl, which presents two interesting and opposite events in metamorphosis (a suppression of metamorphosis through the process of neoteny and its induction by thyroid hormone), is a promising model for the comparative study of postembryonic development at the molecular level in vertebrates (8, 54). Genetic analysis suggests that metamorphic failure in laboratory axolotl is associated with a major locus that is not TRα or TRβ (55). Interestingly, a more recent paper suggests that TRα may contribute to variations in metamorphic timing in wild-caught axolotls, suggesting the possibility of a minor effect of TRs on metamorphic failure (55). In addition, the genetic data indicate that the mechanistic basis of metamorphic failure is different between wild-caught and laboratory axolotl, suggesting that it may have more than one mechanistic basis (56, 57). Given this complex pattern for metamorphic failure for *Ambystoma*, it is likely that several factors have to be taken into account to fully explain the common occurrence of neoteny in these animals. In addition, the axolotl is a promising model to study how the action of a hormone, namely TH, can evolve in different species. For example, the isolation of TH-regulated genes from axolotl tails using a subtractive screening procedure and analysis of the regulation of these genes compared with the regulation of their homologs in *Xenopus* would allow insight into why the urodeles tail is refractory to the action of TH. Our results, although still preliminary, suggest that the axolotl tail can respond to TH, but that the main differences occur at the level of target genes.

Acknowledgments

We thank Hector Escriva, Michael Schubert, and Randal Voss for critical reading of the manuscript; Julien Fellah, Véronique Laurens, and Yannick Andreol for providing animals; Jean-Paul Chaumel for taking care of the animals; and Carl Seguin for the generous gift of the axolotl cDNA library.

Received July 21, 2003. Accepted October 1, 2003.

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This work was supported by Ministere de l'Education Nationale de la Recherche et de la Technologie, Centre National de la Recherche Scientifique, Association pour la Recherche Contre le Cancer, Fondation pour la Recherche Médicale, La Ligue Regionale Contre le Cancer du Rhône et de la Loire and HRA-PHARMA.

R.S. is the recipient of a fellowship from the Ligue Nationale contre le Cancer and the Fondation pour la Recherche Médicale.

O.M. is the recipient of a fellowship from Ministere de l'Education Nationale de la Recherche et de la Technologie and ARC.

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