A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis

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The expression of the thyroid hormone (TH) receptor genes α (TRα) and β (TRβ) in *Xenopus laevis* begins after the embryo hatches. The TRα mRNA increases throughout the premetamorphosis stage of tadpole development, is maximal by prometamorphosis, and falls after climax of metamorphosis to a lower level in frogs. The TRβ mRNA is barely detectable during premetamorphosis. In synchrony with the onset of endogenous TH synthesis by the thyroid gland (prometamorphosis), the level of TRβ mRNA rises in parallel with endogenous TH, reaching a peak at the climax of metamorphosis (stage 61) and drops to ~10% of its peak level after metamorphosis. As suggested by this correlation, exogenous TH up-regulates TRβ mRNA as much as 20-fold during premetamorphosis, whereas TH up-regulates TRα mRNA by ~2-fold during the same period. Up-regulation of TRβ mRNA is the earliest response to exogenous TH by competent tadpoles yet detected.

**[Key Words: Xenopus, metamorphosis; thyroid hormone receptor genes; transcription]**

Received July 18, 1990; revised version accepted August 31, 1990.

Amphibian metamorphosis is divided into three developmental periods (Dodd and Dodd 1976). Premetamorphosis refers to the development and growth of the tadpole that occurs before formation of a functional thyroid gland. In *Xenopus laevis*, premetamorphosis ends at about stage 54 (Nieuwkoop and Faber 1956) when the developing thyroid gland begins to secrete thyroid hormone [TH] (Leloup and Buscaglia 1977), the known biological effector of metamorphosis. An orderly succession of morphological changes occurs during the next stage, prometamorphosis [NF55–57], the most obvious of which is limb development. The front legs erupt from the opercular fold, and the shape of a miniature frog is almost complete by the third stage, or climax, when the concentration of endogenous TH is maximal. The final change after stage 62 is a rapid resorption of the tail. These transitions that affect almost every tissue are dependent on the concentration of TH [Kollos 1961], which itself is controlled by a feedback loop connecting the pituitary, the hypothalamus, and the thyroid gland [Etkin 1968].

We began an investigation of this complex process by cloning and characterizing members of the two known thyroid hormone receptor [TR] families—α and β (Yaoita et al. 1990). In *Xenopus* there are two TRα (A and B) and two TRβ (A and B) genes. Here, we report the level of TRα and TRβ mRNAs during *Xenopus* development and we describe the extent to which their mRNA levels correlate with metamorphosis.

**Results**

**An assay for the TRα and TRβ mRNAs**

The major components of both TRα and TRβ mRNAs are ~10 kb in size; they are rare and difficult to detect by traditional Northern blot analysis [Fig. 1]. The isolation of poly(A)⁺ RNA does not improve the yield of these mRNAs very much, perhaps because of their large sizes. We developed an assay in which the mRNA is converted into cDNA by reverse transcriptase primed by a specific antisense oligonucleotide. The resulting cDNA is electrophoresed, blotted, and hybridized with a radioactive probe, as for a standard Northern blot. This modified primer-extension method has several merits. First, the radioactive signal is from 3- to 10-fold greater than that obtained from a Northern blot of an equivalent amount of mRNA. We have found this to be true for the mRNAs for TRα and TRβ and to varying extents for other mRNAs. The augmented signal is due partly to the greater retention of DNA than RNA on filters and partly to the difficulties of working with very high-molecular-weight RNA and keeping it intact. The primer extension method, using a primer 3' to the termination codon, converts a large 10-kb mRNA to a cDNA of ~1.8 kb [Fig. 1]. Because the 5'-untranslated region (UTR) and the coding sequence comprise ~2 kb [Yaoita et al. 1990], the remaining 8 kb of these mRNAs must be located in the 3' UTR.

The TRα reverse transcripts are present as a promi-
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Figure 1. Comparison of Northern blotting and modified primer extension methods for determining transcripts of TRα (left) and TRβ (right) mRNAs. Either poly[A]+ RNA (4 μg) or cDNA synthesized from 10 μg (TRα) or 7 μg (TRβ) of total RNA isolated from stage 58 tadpoles was loaded in each lane. (Upper and lower dots) The positions of 28S and 18S rRNAs, respectively.

The developmental expression of TRα and TRβ genes

Expression of the TRα and TRβ genes was measured by the primer-extension method using as template total RNA from whole tadpoles at various stages (Figs. 2 and 9, below). TRα and TRβ mRNA were not detected in oocytes, eggs, or embryos until after hatching. TRα mRNA increases from stage 38 throughout premetamorphosis, reaching a peak by early prometamorphosis (stage 56); it remains elevated until climax and then decreases to about half of its peak value in frog liver. TRβ mRNA is low throughout premetamorphosis, rises to a peak at climax, and then falls. The expression pattern of TRβ mRNA follows closely the concentration of endogenous TH (Leloup and Buscaglia 1977) (see Fig. 9, below). There is a subtle change in the pattern of cDNA bands synthesized from TRβ mRNA as development proceeds. The slower-moving of the two cDNA fragments in the upper doublet of bands predominates during premetamorphosis. At about stage 52, the faster-moving cDNA band is detectable. Transcripts of both TRβ genes contribute about equally throughout development (see Fig. 6, below).

Exogenous TH up-regulates TRβ mRNA

The close correlation of TRβ mRNA with endogenous TH formation by tadpoles suggested that the level of TRβ mRNA is regulated, in part, by TH concentration. Two-day treatment of embryos from stage 40 with 3,5,3′-triiodo-L-thyronine (T3) results in the premature appearance of the faster-moving of the two cDNAs in the upper doublet synthesized from stage 44 RNA, and showed fourfold up-regulation of TRβ mRNA [Fig. 3]. Stage 48 tadpoles treated previously for 6 days with T3 had their TRβ mRNA up-regulated to the level of control stage 61 Xenopus at climax. After stage 48 and throughout premetamorphosis, a 1-day treatment induces a maximal response (Fig. 4). Early morphological changes, such as the growth of hind limbs, are observed at stages 47–48 after continuous exposure to T3 and ~1–2 days after up-regulation of TRβ mRNA has occurred.

In a competent tadpole during premetamorphosis, TRβ mRNA up-regulation is detectable 8 hr after the addition of T3 to the water, it is maximal after 16 hr (Fig. 4). A much lower extent of TRα mRNA up-regulation (about two- to threefold) also occurs by 8 hr after TH treatment. Another correlation of TRβ mRNA up-regulation with metamorphosis can be shown by removing exogenous T3 several days after its administration during the premetamorphosis period. The induced morphological changes are arrested, and the level of TRβ mRNA drops [Fig. 5]. Thus, continuous administration of T3 is required both for TRβ mRNA up-regulation and the induction of morphological changes. Up-regulation of both TRβA and TRβB mRNAs by exogenous T3 can be demonstrated in competent tadpoles [Fig. 6]. Once metamorphosis has proceeded to climax (stage 62), exogenous T3 no longer up-regulates TRβ mRNA, presumably due to the high levels of endogenous T3. In frogs, the level of TRβ mRNA [and T3] is lower than the peak concentration at climax, and some up-regulation occurs with exogenous T3 [Fig. 6].

Expression of TR in tail and hind limbs

During prometamorphosis, the hind limbs develop with no change in tail size. At climax (stage 63), the tail regresses rapidly at a time when hind limb development is complete. We compared the TR mRNA content in hind limbs and tail at stages 58 and 63 (Fig. 7). Both TRα and...
TRβ mRNAs are elevated in tissues that are in the act of transforming, that is, hind limbs at stage 58 and tail at stage 63.

Correlation of TR mRNAs with metamorphosis of neotenic amphibians

Some neotenic salamanders such as *Necturus maculosus*, the mud puppy, remain larval all their life and cannot be induced to metamorphose, even with large doses of exogenous TH [see Dent 1968]. Others, such as *Ambystoma mexicanum*, the axolotl, usually remain larval in nature but can metamorphose spontaneously or be induced to transform with exogenous TH [Kühn and Jacobs 1989]. Morphological change of a TH-responsive urodele such as the axolotl is more limited than it is in anurans, but one major change is gill resorption.

We measured the levels of TR mRNAs in extracts of gills of *Necturus* and axolotl as a response to exogenous T3 treatment. Northern blots probed with cloned *Xenopus* TR cDNAs did not detect TR mRNAs. In the absence of sequence information, we primed cDNA synthesis from total RNA with oligo(dT) and electrophoresed, blotted, and hybridized the cDNAs with various *Xenopus* TR cDNAs [Fig. 8]. This experiment will only succeed if there is a poly[A] priming site reasonably close to the coding region of the mRNAs. The results show substantial hybridization with the cDNA synthesized from axolotl but not *Necturus* cDNA. It further shows up-regulation of axolotl TR mRNA of about fourfold following T3 injection. *Xenopus* TRα and TRβ cDNA probes give similar results, so we cannot distinguish which family of TR transcript is being detected by these heterologous probes. The result correlates TR mRNA content with the known responsiveness of axolotl to T3 with the caution that this method of measuring heterologous mRNAs of unknown sequence may miss an abundant relevant mRNA altogether.

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**Figure 2.** The expression of TR genes during *Xenopus* development. The cDNA synthesized from 10 μg whole tadpole RNA at each stage was loaded in each lane and analyzed using TRα (upper panel) and β probes (lower panel). These filters were rehybridized with the RP28 probe to standardize the amount of nucleic acid loaded in each lane, and the results are shown below the TRα and TRβ panels. The first and second stage 48 samples were from 9- and 13-day-old tadpoles, respectively.

**Figure 3.** The response of TR genes to T3. Embryos and tadpoles were treated with 5 nM T3 for varying periods before RNA was extracted [labeled T3 (d)]. Ages of embryos or tadpoles from the time of fertilization to the time of RNA extraction are represented in days [d]. A dash means no treatment with T3. A total of 10 μg of whole tadpole RNA was converted to cDNA and one-third of it was loaded in each lane. The arrow marked by M indicates the time when the first morphological change was observed (i.e., 8-day-old tadpole treated for 5 days). The upper panel is probed with TRα; the lower panel, with TRβ.
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Figure 4. The accumulation of TR mRNAs after T₃ treatment. Stage 52 tadpoles were treated in 5 nM T₃ water for various times in hours (HR) before RNA extraction. These RNAs were analyzed as in Fig. 3.

Discussion

Correlation of TRα and TRβ mRNAs with metamorphosis

The conservation of two distinct but closely related families of TH receptors, TRα and TRβ, from mammals to chickens has now been extended to an amphibian, *Xenopus*. To date, the only clue that the two kinds of receptors may have distinct functions comes from correlation of their patterns of expression. In chicken, TRα mRNA expression is described as ubiquitous and constitutive, while TRβ mRNA increases at developmental periods and in specific tissues when the embryo is known to be sensitive to TH (Forrest et al. 1990). Expression of TRβ mRNA in rat brain by virtue of its cell type distribution and developmental pattern has suggested a developmental function (Bradley et al. 1989).

There is one reported example of an alternatively spliced variant of TRβ (called rTRβ2) that is expressed only in the pituitary of the rat (Hodin et al. 1989). These reports correlate TRβ more than TRα with developmental control and tissue-specific expression. Recently, we demonstrated that there are two *Xenopus* TRβ genes, and their mRNAs undergo complex alternative splicing of as many as eight exons in the 5'-untranslated regions (Yaoita et al. 1990). This alternative splicing yields at least two different proteins at the amino terminus for both TRβA and TRβB. Furthermore, the proteins vary at the same splice junction as the tissue-specific rat TRβ2 variant. We have not yet demonstrated tissue specificity for this alternative splicing, but there is a developmental change in mRNA complexity that could be explained by RNA splicing. A single reverse-transcribed cDNA band at early stages changes to two closely approximated bands after stage 52 (Figs. 2—4). This latter pattern is induced by TH (Fig. 3).

The pattern of developmental change of TRβ mRNA (Fig. 9) correlates with metamorphosis, especially the striking up-regulation of TRβ mRNA after administra-
Regulation of a human keratinocyte-specific gene

St b8

The satisfactory conclusion that *Necturus* lacks one or all classes of TR mRNAs is tempered by the lack of a suitable control that would prove that this method can detect *Necturus* TR mRNAs should they exist.

Developmental Northern blots were reported by Baker and Tata (1990) throughout *Xenopus* development by use of chicken TRα and TRβ cDNA probes. Hybridization with the heterologous TRα probe from chicken detected mainly an abundant 4.2-kb RNA, which we have not detected; therefore, the hybridization assay of these investigators is measuring something different from our own. Their TRβ mRNA analysis was limited to slot blot hybridization, but showed greater hybridization at stage 61 than at stage 58 (as we have found). However, they report that TR mRNA levels were unaffected by TH administration.

**Figure 7.** Expression of TR genes in hind limbs and tail at two stages. Total RNAs (10 μg) extracted from hind limbs (HL) and tail (T) of stage 58 and 63 tadpoles were used to synthesize cDNAs. After the analysis of TRα (upper) and TRβ (lower) gene expression, these filters were rehybridized with the RP28 probe (below the TRα and TRβ panels).

Up-regulation of TRβ mRNA

Hormones can influence gene expression by transcriptional or post-transcriptional control mechanisms. An example of a ligand up-regulating the mRNA encoding its own receptor is the transcriptional up-regulation of the human β-retinoic acid receptor by retinoic acid ([de Thé et al. 1989, 1990]). Estrogen can both stabilize vitellogenin mRNA and regulate its transcription ([Shapiro et al. 1984]). Estrogen also up-regulates estrogen receptor mRNA ([Barton and Shapiro 1988]). TH has been reported to down-regulate TRα mRNA in the rat ([Strait et al. 1990]) and to down-regulate the pituitary-specific TRβ2 ([Hodin et al. 1989]).

There have been several studies correlating the binding of radioactive T3 to various tadpole tissues during metamorphosis as a measure of functional re-

**Figure 8.** Expression of TR genes in gills of neotenic salamanders. The cDNAs were prepared from 10 μg of total RNA from gills of axolotl and *Necturus* control or T4-injected animals. The cDNAs were electrophoresed, blotted, and hybridized to a *Xenopus* TR8 probe (upper panel) and then to the RP28 probe (lower panel). In both cases, the filter was washed with 2× SSC at room temperature twice and then with 0.1× SSC−0.1% SDS at 50°C for 10 min.
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Figure 9. A summary of the developmental expression of TRα and TRβ genes throughout embryogenesis, tadpole growth, and metamorphosis. Stages of development of X. laevis embryos and tadpoles [Nieuwkoop-Faber 1956] with representative pictures are plotted against the amounts of TRα (كرة) and TRβ (מ) cDNAs and T3 (ד) [Leloup and Buscaglia 1977] in arbitrary units, calling the amount at stage 61 as 1.0. Quantitation of band density (Fig. 2) was measured by densitometry using preflashed film. Values were corrected for the abundance of the RP28 mRNA, which was assumed to be constant throughout development (after hatching). The levels of TRβ cDNA (מ) in tadpoles up-regulated by exogenous T3 during premetamorphosis are also shown. An arrow (מ) indicates the time when the first morphological change induced by T3 was detected.

Competence and the first step of the metamorphosis cascade

We have presented no functional evidence that TRα and/or TRβ are intermediates in metamorphosis. These studies are only correlative in nature and to date have only measured mRNA levels, not the proteins that they encode. Even these mRNA measurements lose detail because mainly total tadpole RNA rather than individual tissues were studied. However, these measurements suggest two obvious insights into the first steps of metamorphosis. The first is a simple explanation for the phenomenon of “competence” [Tata 1968], that is, the ability of growing tadpoles to respond precociously to exogenous TH. Competence correlates with the synthesis of TR mRNAs, especially the ability of exogenous T3 to up-regulate TRβ mRNA. Second, we note that TRα mRNA accumulates during premetamorphosis but TRβ mRNA remains low until endogenous T3 is synthesized. It has been demonstrated that TR proteins repress TH-responsive genes in the absence of TH [Damm et al. 1989; Forman et al. 1989]. By analogy, perhaps the cascade of metamorphosis is repressed during premetamorphosis in a competent tadpole by TRα protein binding to response elements in the TRβ genes. Hypothetically, this repression would be relieved by TH during premetamorphosis up-regulating the expression of TRβ, which, in turn, activates (or represses) the downstream genes in the cascade. We imagine that the first downstream set of genes encodes tissue-specific transcription factors, which then activate the many genes whose products ac-
count for the global morphological and biochemical changes that characterize the transition from tadpole to frog.

Materials and methods

Animals

*Xenopus laevis* embryos were reared in dechlorinated tap water (frog water) and fed with nettle powder. Staging of embryos was according to the table of Nieuwkoop and Faber [1956]. Metamorphosis was induced by adding 5 nM $T_3$ to frog water. The frog water was changed daily. Adult axolotl were obtained from the axolotl center at Indiana University. *Neoturus* were purchased from Charles Sullivan Company. Axolotl and *Neoturus* were injected intraperitoneally once with 0.3 $\mu$g of thyroxine/gram of body weight (Norris and Platt 1974) and maintained in 26 $\mu$g thyroxine [$T_4$] for 2 days before total RNA was extracted from their gills.

RNA isolation, blotting, and hybridization

A single mating provided tadpoles for each developmental series. RNA was extracted from 10–50 pooled tadpoles at each time or developmental stage. Whole tadpoles or tissues were homogenized in guanidinium thiocyanate [Chirgwin et al. 1979]. The homogenate was centrifuged briefly to remove debris and aggregates. RNA was separated from DNA and proteins by cesium chloride centrifugation and then extracted with phenol and chloroform. The poly[A]$^+$ RNA fraction was obtained by passing total RNA over an oligo(dT)-cellulose column and eluting with a low-salt buffer. RNAs were denatured in the presence of glyoxal and DMSO and subjected to electrophoresis in an agarose gel. RNAs were blotted onto Nytran (Schleicher & Schuell), UV cross-linked, and baked in a vacuum oven at 80°C for 2 hr. The filter was hybridized with a labeled probe and washed with two changes of 2x SSC at room temperature, 0.1 $\times$ SSC–1% SDS at 65°C for 1 hr, and two changes of 0.1 $\times$ SSC–0.1% SDS at 65°C for 15 min each. In our experience, Nytran filters have a high retention efficiency for RNA.

Modified primer extension assay

The cDNA was synthesized from total RNA by avian reverse transcriptase primed with gene-specific antisense oligonucleotides: 5’-[1346]ATTGTCTTCTTTAGGATAT(1324)-3’ [for TRa] or 5’-[1341]GCCAAACACACTGCTTGT(1324)-3’ [for TRp]. Numbers in parentheses locate the positions of the antisense primers, counting the adenosine of the initiation codon in the TRa gene as one, or the guanine adjacent to the “changing point” of TRp gene, which is located at the 5’ end of the first zinc finger in the TRp gene [Yaota et al. 1990]. Although there are two nucleotide differences in this region between TR8A and TR8B, this oligonucleotide primes cDNA synthesis from both mRNAs with the same efficiency under these conditions [data not shown]. The cDNA was extracted in phenol and chloroform, precipitated with ethanol, denatured by DMSO and glyoxal, fractionated by agarose gel, and transferred to Nytran filters. The hybridization procedure is described above.

*Xenopus* TRa and TRp probes were prepared from cDNA inserts [556–1212] for TRaB and [122–1428] for TRB A cDNA clones by nick-translation or random primer labeling methods. The former fragment contains the junction region [between the DNA-binding and thyroid hormone-binding domain] and the thyroid hormone-binding domain, the latter fragment contains the same regions but, in addition, the second zinc finger and part of the 3’-untranslated region. The TRa and TRp probes hybridized to TRaB and TRaB and TRpB, respectively, in Southern blots of genomic DNA. The TRaB probe does not cross-hybridize with the TRpB probe by use of stringent hybridization conditions. The TRaB- and TRpB-specific probes were prepared from the first zinc finger fragment of TRaB and TRpB genes, respectively, by a repetitive polymerase labeling method [Yaota et al. 1990].

The amounts of RNA from each embryo stage were standardized by rehybridizing with a cDNA [PR28] that encodes a *Xenopus* RNA that is constitutively expressed after stage 35 (Shi and Brown 1990). The primer extension reaction used to prepare TR cDNA does not degrade the PR28 mRNA, and the level of this mRNA is not influenced by $T_3$. The PR28 cDNA also hybridizes to *Neoturus* and axolotl mRNA (Fig. 8). PR28 has not been sequenced. Band densities were traced with a densitometer from the autoradiogram of a preflashed film.

Acknowledgments

We thank our colleagues for their many helpful criticisms. This research was supported, in part, by grants from the National Institutes of Health and the Lucille P. Markey Charitable Trust. The publication costs of this article were defrayed, in part, by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.

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*Genes Dev.* 1990, 4:
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