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# *Hormonal Signalling during Amphibian Metamorphosis*

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Metamorphosis is a most dramatic example of extensive morphological, biochemical and cellular changes occurring during postembryonic development. The process, best known in some amphibia and insects, is initiated and sustained by hormonal signals which serve to put the developing organism in touch with environmental cues. The same hormones that regulate metamorphosis in amphibia also influence various late developmental processes in other vertebrates including man. Although other hormones can modulate the process, amphibian metamorphosis is obligatorily regulated by thyroid hormone which, after an early developmental stage, is capable of initiating metamorphosis precociously. Each tissue of the tadpole responds differently to the hormone, ranging from altered gene expression, morphogenesis, tissue re-structuring and extensive cell death, according to a developmental programme that is in place before the thyroid gland begins to secrete the hormone. The key element determining the response to the hormone is the thyroid hormone receptor which is a member of the nuclear receptor superfamily of ligand-activated transcription factors. As in all vertebrates, there are two thyroid hormone receptor genes, TRα and TRβ, whose concentration in the tissues is directly modulated by the hormone itself. Biochemical and *in situ* techniques have shown that the amount of TRβ mRNA and protein is elevated 50-100 times during metamorphic climax. This phenomenon of autoinduction of receptor is also seen with developmental or inductive processes regulated by other hormones acting through nuclear receptors. It is possible that receptor autoinduction may be a pre-requisite for hormonal response. Recent molecular and cell biological studies have suggested that nuclear receptors function as multimeric complexes with other proteins within chromatin to regulate the structure of the chromatin and thereby determine the transcription of the receptor-specified target gene. First studies indicate that this may be so for thyroid hormone regulated transcription during amphibian metamorphosis. While several mechanisms still remain to be elucidated, it is clear that amphibian metamorphosis has proved to be an excellent model for understanding the role of hormonal signalling during postembryonic development in vertebrates.

*Key* **W***ords: Hormones, Amphibia, Thyroid hormone, Postembryonic development, Nuclear receptors, Thyroid hormone receptor, Programmed cell death*

## *Introduction*

Metamorphosis is among the most dramatic changes occurring during postembryonic development in vertebrates and invertebrates. From its Greek derivation it simply means a change in form or shape. Biologists had for long been intrigued by the dramatic and apparently spontaneous transformation of a tadpole into a frog or an insect larva or pupa into the adult, both classical examples of metamorphosis. Early studies had indicated that these morphological changes were accompanied by profound alterations in chemical composition and biochemical functions. Unlike early embryogenesis, the initiation and progression of metamorphosis is obligatorily controlled by highly specific hormones. It is this discovery in the last century that this postembryonic developmental process is dependent on endocrine secretions that has made it possible to understand how metamorphosis was brought about

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and regulated. This review is restricted to amphibian (almost exclusively anuran) metamorphosis. Before considering the molecular mechanisms underlying hormonal signalling controls that regulate this dramatic process of postembryonic development, it is worth taking into account the major characteristics of the process. The reader will find much useful detailed information in several volumes specifically dedicated to the subject of metamorphosis (Weber 1967, Etkin & Gilbert 1968, Gilbert & Frieden 1981, Gilbert et al. 1996, Shi 1999).

### *Amphibian Metamorphosis is Obligatorily Controlled by Hormones*

A salient, and evolutionally highly conserved, feature of insect and amphibian metamorphosis is that the process is obligatorily initiated and sustained by hormonal signalling. In amphibia it has been known since the discovery by Gudernatsch in 1912 of the precocious induction of metamorphosis in frog tadpoles that the process is under the control of thyroid hormone (TH). With the recognition of the central role played by the hypothalamus-pituitary-thyroid axis in vertebrates, the link between environmental signals and the initiation of metamorphosis could also be traced to the central nervous system through the intermediary of the hypothalamic hormone CRF (corticotrophin releasing factor) and TSH (thyrotrophic hormone) made in the pituitary (see figure 1). The earlier notion of TRH (TSH releasing hormone) playing a role in the facilitation of TH action (Kikuyama et al. 1993) has been invalidated and it seems that CRF plays the same role (Denver 1996). The concept of a positive feedback loop between thyroid hormone (TH) and TSH at the onset of amphibian metamorphosis, first proposed by Etkin, in 1963 (reviewed in Etkin & Gilbert 1968) is in marked contrast to the general notion of negative feedback loops operating between the hypothalamus-pituitary axis and peripheral endocrine glands. Recently Huang et al. (2001) have demonstrated the existence of a negative feedback loop between TH and TSH and have explained the onset of this feedback loop as a function of the action of type II iodothyronine deiodinase, the enzyme which converts thyroxine to the active hormone $T_3$. It still remains to be explained how a tadpole goes

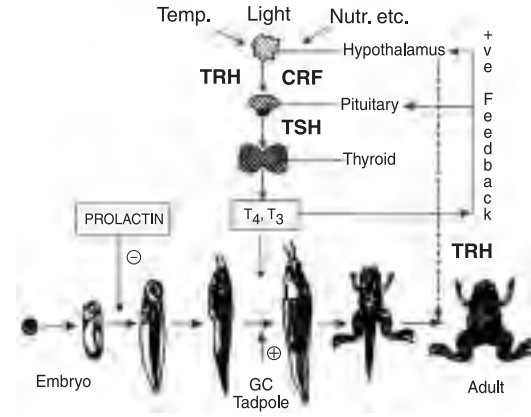


*Figure 1* Scheme depicting the hormonal regulation of amphibian metamorphosis in the frog tadpole.
Environmental factors trigger off the sequential release of hypothalamic (CRF) and pituitary (TSH) hormones which stimulate the thyroid gland to synthesize and secrete thyroid hormones ($T_4$, $T_3$). Although not fully understood at the cellular level, exogenous prolactin prevents or slows down the action of $T_4$ and $T_3$. Other hormones (glucocorticoids, CRF) are also known to modulate the action of thyroid hormones. Abbreviations: *CRF*, corticotrophin releasing factor; *TSH*, thyroid stimulating hormone; $T_4$, $T_3$, L-thyroxine and 3,3',5-triiodo-L-thyronine; *GC*, glucocorticoid hormone; +ve, positive; + = stimulation; - = inhibition. The role of TRH is questionable and is hence shown as a broken line with an interrogation mark.

through "metamorphic climax", whereby both TSH and TH are secreted at a rapidly accelerated rate. The answer may lie in the involvement of other hormones, besides TSH, in regulating the synthesis and secretion of TH. Although thyroid hormone is the only obligatory signal for the initiation and completion of amphibian metamorphosis, other hormones and factors can modulate the onset and progression of metamorphosis. These include glucocorticoid hormone, as well as CRF and adrenocorticotrophin (ACTH) which can accelerate TH-induced metamorphosis, both in intact tadpoles and isolated tissues (Kikuyama et al. 1993). In this context, it is important to bear in mind that CRF, ACTH and corticosteroids can both accelerate and slow down metamorphosis, depending on factors such as environmental conditions, nutrition and developmental stage of the tadpole (Denver 1996, Hayes 1997). Amphibian metamorphosis is well known to be sensitive to photoperiodicity and circadian rhythms. It is slowed down in tadpoles placed in the dark or by exogenous melatonin, which is principally produced in the pineal gland (Wright et al. 1991).

Many investigators have reported that exogenous mammalian and amphibian prolactin (PRL) will prevent both natural and TH-induced metamorphosis in many different species of tadpoles (Kikuyama et al. 1993, Tata 1997, 1998). Intriguingly, circulating PRL levels tend to increase in *Xenopus* and bullfrog tadpoles during and after metamorphosis, suggesting other possible roles for this hormone in adults not yet fully defined. Nevertheless, PRL has been shown to inhibit both the $T_3$-induced growth and differentiation of the *Xenopus* tadpole limb bud and the regression of the tail directly in organ culture (Tata et al. 1991). Very recently, Shintani et al. (2002) have proposed a novel explanation for the antimetamorphic action of PRL (and growth hormone) in some tissues of the *Xenopus* larva. These authors suggest that these two hormones upregulate the expression of type III iodothyronine 5-deiodinase, an enzyme that inactivates TH, in a highly tissue-specific manner in the tail but not in the liver. There may be other explanations also for the mechanism of action , but although this action of PRL as physiologically meaningful in the whole larva is still debatable (Huang & Brown 2000), PRL can still be a useful tool in exploring the mechanism of action of TH in regulating amphibian metamorphosis.

In amphibia, the initiation and maintenance of an uninterrupted larval-adult transition requires the appearance and maintenance of high circulating level of thyroid hormone (Kaltenbach 1968, Leloup & Buscaglia 1977, Shi 1999, Tata et al.1993). Simple exposure of amphibian tadpoles to TH can precociously induce normal metamorphosis. Conversely, withholding the hormone by surgical or chemical ablation of the thyroid gland will prevent further development or metamorphosis until such time as the hormone is replaced (Kaltenbach 1968). Precocious induction of metamorphosis by simple hormonal manipulation as an experimental approach has contributed greatly to our understanding of the physiology and biochemistry of postembryonic development.

### H*ormonal Control is Direct,* L*ocal and Tissue-specific*

An important feature of the action of metamorphic hormones is that it is not systemic but direct and local. Early studies on local application of hormone to different parts of the premetamorphic frog tadpole established this point (Etkin & Gilbert 1968). For example, work done by Kaltenbach showed that when L-thyroxine ($T_4$) dissolved in cholesterol and applied as a pellet (to prevent its rapid diffusion) was to one side of the tadpole tail fin and the base alone to the other, only the site at which $T_4$ was applied underwent local regression (see Kaltenbach1968). Similar results were obtained when only the tadpole eye to which $T_4$ was applied locally contained rhodopsin (adult) and not the control eye which had porphyropsin (larval) as the visual pigment. The porphyropsin-rhodopsin transition, along with that of larval-adult haemoglobin, are among the major biochemical processes of gene switching during amphibian metamorphosis (Wald 1981).

Equally convincing proof of local effects is provided by organ culture of larval tissues exposed to the metamorphic hormone. In the mid-1960s it was possible to show that triiodothyronine ($T_3$), the major thyroid hormone, added to organ cultures of early pre-metamorphic *Xenopus* tadpole tails caused them to undergo regression with the same morphological and biochemical criteria seen during natural metamorphosis (Tata 1966). Later, organ cultures demonstrated the local and direct effects of $T_3$ for both the cell death response actions of $T_3$ and the anti-metamorphic effects of prolactin (Tata et al. 1991).

Another important feature of local effects of hormones worth considering is the fact that the tissue responses are independent of the location of the target cells. Early transplantation experiments, such as those carried out by Schwind in the 1930s made this point quite clearly (see Kaltenbach 1968). For example, tadpoles in which the tail was transplanted just below the head and then allowed to develop naturally, or exposed to exogenous thyroid hormone, the transplanted tail proceeded to regress just as the normal tail although the tissue surrounding the transplant did not. Conversely, the eye transplanted on the tail underwent biochemical differentiation and morphogenesis to the adult phenotype whereas the surrounding tail tissue regressed and ultimately disappeared. These demonstrations of direct hormonal responses and tissue positional independence point to the important

fact that the hormone merely serves to initiate a dormant developmental programme laid down at an early stage in postembryonic development.

Much information has accumulated in the last seventy years about the diversity and extensiveness of morphological and biochemical changes in amphibian larva in response to thyroid hormone (Weber 1967, Etkin & Gilbert 1968, Gilbert et al. 1996). Table 1 lists some of the well-known responses of various amphibian larval tissues to TH. Although different genes constitute the responses of different cell types, there is a common feature underlying the hormonally regulated postembryonic developmental process, namely the acquisition of the adult phenotype. An injection of TH to early tadpoles leads to the premature activation of the developmental programme. In all instances, the biochemical changes are preceded by an early burst of RNA synthesis, which is essential for the later changes in phenotype as shown by experiments with transcriptional inhibitors. This is illustrated by the induction by TH of newly synthesized mRNA encoding urea cycle enzymes (carbomyl phosphate synthetase, ornithine transcarbamylase, arginosuccinate synthetase) and serum albumin in early bullfrog (*Rana catesbeiana*) tadpole liver (Weber 1967, Cohen 1970, Atkinson et al. 1996).

The second striking feature of hormonally regulated postembryonic development is that different tissues, or groups of cells within the same tissue, can exhibit different hormonal responses, which range as widely as *de novo* morphogenesis, functional reprogramming and total tissue regression. To consider a few examples, the amphibian larval brain is a major hormonal target with a wide variety of changes taking place during metamorphosis in both its anatomical and functional characteristics. Similarly, the skin, pancreas and liver undergo genetic reprogramming leading to the acquisition of new morphological and biochemical characteristics, such as the appearance of digestive enzymes in the pancreas, urea cycle enzymes and serum albumin in the liver and the keratinisation of the larval skin are good examples. Among the most dramatic morphological and biochemical changes are the almost simultaneous emergence of limbs and total or substantial loss of larval tail, gills and the intestine. Indeed, there are few, if any, larval cells that escape the impact of thyroid hormone.

*Table 1 Diversity of morphological and biochemical responses during thyroid hormone-induced amphibian (anuran) metamorphosis*

| Tissue | Response | |
|---|---|---|
| | Morphological | Biochemical |
| Brain | Re-structuring, axon guidance, axon growth, cell proliferation and death | Cell division, apoptosis and new protein synthesis |
| Liver | Re-structuring, functional differentiation | Induction of urea cycle enzymes and albumin; Larval to adult haemoglobin gene switching |
| Eye | Re-positioning; new retinal neurones and connections; lens structure | Visual pigment transformation (porphyropsin - rhodopsin); beta-crystallin induction |
| Skin | Re-structuring; skin granular gland formation; keratinization and hardening; apoptosis | Induction of collagen, 63 kDa (adult) keratin and magainin genes; induction of collagenase |
| Limb bud, lung | De novo formation of bone, skin, muscle, nerves, etc. | Cell proliferation and differentiation; chondrogenesis |
| Tail, gills | Complete regression | Programmed cell death; induction and activation of lytic enzymes (collagenase, nucleases, phosphatases, matrix metalloproteinases); lysosome proliferation |
| Pancreas, intestine | Major tissue re-structuring | Reprogramming of phenotype, acquisition of new digestive functions, induction of proteases, fatty acid binding protein, stromelysin-3 |
| Immune system | Re-distribution of cell populations | Altered immune system and appearance of new immunocompetent components |
| Muscle | Growth and differentiation; apoptosis | Induction of myosin heavy chain |

*See Tata 1998; Atkinson et al. 1996; Shi 1999 for further details.*

Thus, tissue-specific differential gene expression is central to hormonal signalling and postembryonic development. It is also worth re-emphasizing that the hormone does not determine the developmental programme but serves to initiate it. Another notable process during metamorphosis is the turnover and replacement of cell populations during metamorphosis is provided by the switch from larval to adult haemoglobin in erythroid cells of *Xenopus* larvae. At one time it was thought that the switch to express adult haemoglobin genes occurred in the same nucleated erythrocyte that carried larval haemoglobin prior to the onset of metamorphosis. However, immunofluorescence with specific antibodies led Weber and colleagues (1996) to demonstrate that the larval haemoglobin carrying cells were progressively replaced by those expressing adult haemoglobin following the onset of metamorphosis.

A large number of genes activated by TH in various larval amphibian tissues during natural and hormone-induced metamorphosis have been identified (Atkinson et al. 1996, Weber1996, Brown et al. 1996, Shi et al. 1996, 1999, Tata 1997, 1998). These include such genes as serum albumin, adult haemoglobin and carbomyl phosphate synthetase in *Xenopus* tadpole liver. In the laboratories of Brown and Shi the analysis of gene switching has been extended to the characterization of genes that are silenced or down-regulated during amphibian metamorphosis. Table 2 lists some of the up- and down-regulated genes in the limb bud (*de novo* morphogenesis), intestine (partial regression) and tail (total cell death) by TH administered to premetamorphic *Xenopus* tadpoles. Many unidentified up- and down-regulated genes are not included in this table. Of particular interest are genes that can be classified as 'early' or 'direct response' genes (activated in the absence of protein synthesis) since it is likely that their products may play a causal role in the cascade of regulatory elements leading to tissue-specific biochemical and morphological changes listed in table 1. Also noteworthy is the fact that many direct response genes encode transcription factors (table 2). Recently, Furlow and Kanamori (2002) have confirmed that the SP1-type zinc finger transcription factor xBTEB1 (*Xenopus* basic transcription element binding protein 1) as a direct response gene and predict that it may play an important role in downstream gene regulation during TH-induced metamorphosis. The most significant in this class of genes are those encoding receptors for TH. Their significance will be discussed later.

From early studies on the activation of the amphibian tadpole's thyroid gland by the cascade of neurosecretory hormones (see figure 1) it had already emerged that metamorphosis would occur soon after this gland begins to secrete thyroid hormone. At the same time, ablation or chemical inactivation of thyroid gland, early in development, followed some time later by administration of exogenous TH clearly established that all the larval tissues were competent to undergo metamorphic changes before the endocrine gland became active. These observations raised the important question as to how early in development would the larval tissues be capable of responding to the

*Table 2 A few examples of up- and down-regulated genes in Xenopus tadpole tissues in response to thyroid hormone ($T_3$)*

| Tissue | No. of genes | | Upregulated direct response genes |
|---|---|---|---|
| | Upregulated | Downregulated | |
| Hind limb | 14 | 5 | Heat shock protein<br>Zn finger (E4.BP4) |
| Intestine | 22 | 1 | NF-1<br>$Na^+/PO_4$—<br>Cotransporter<br>bZip (E4BP4)<br>Stromelysin-3 |
| Tail | 35 | 10 | Zinc finger (BTBEB1)<br>Stromelysin-3<br>Iodothyronine deiodinase |

*See Shi 1999, Brown et al. 1996 and Furlow and Kanamori 2002 for further details.*

metamorphic hormone, a question highly relevant to developmental expression of their receptors. A systematic study in which different stages of *Xenopus* embryos and tadpoles were exposed to TH gave a clear-cut answer to the above question (Tata 1968). Measurement of a number of morphological and biochemical responses revealed that the competence to respond to $T_3$ was established as early as stage 44 or 45, i.e. a week after fertilization of the egg. By stage 47 or 48, which is about 2 weeks later, all the responses are more clearly evident. Natural metamorphosis begins at stage 53 or 54 which, depending on external conditions, can be about 2 months after fertilisation, which is also when TH is first detectable in the tadpole blood. Subsequently, the rapid build-up and decline in circulating TH temporally matches the onset and completion of natural metamorphosis. Thus, the acquisition of the response can be seen several weeks before the detection of the hormone. Similar observations of early competence of response have been recorded for insect metamorphosis (Cherbas & Cherbas 1996, Riddiford 1996).

The above issue of early developmental competence in amphibia is particularly well illustrated by using molecular markers that specify the adult or post-metamorphic phenotype. Serum albumin is a major hepatic gene that is switched on during thyroid hormone-induced metamorphosis in all anurans (table 1). If early pre-metamorphic *Xenopus* tadpoles (at stages up to 45) are exposed to $T_3$ for 3 days, the albumin gene is not expressed in response to the hormone. However, at later stages, as for example at stage 48, the albumin gene is precociously induced by the hormone (Baker & Tata 1990). These findings lead to the conclusion that the functional receptor must be in place considerably before the hormonal signals impinge on the tissues during normal development and highlight the importance of considering the expression of receptor genes before and during metamorphosis.

*Developmentally-Programmed Cell Death is an Important Feature of Metamorphosis*

Upon the onset of natural or hormone-induced metamorphosis the amphibian larva undergoes a substantial loss of cells in many tissues which continues until the process is completed. This loss of cell number, as well as the total mass of the organism, can be manifested as the loss of entire organs, as is the case for tadpole tail and gills. This postembryonic developmental cell death is not restricted to total or externally visible tissue regression but can be quite extensive in internal organs that are morphologically and functionally re-structured, such as the brain, intestine and pancreas. These tissues will undergo further development and can exhibit substantial cell proliferation as metamorphosis reaches completion.

Earlier studies to explain thyroid hormonal induction of tissue regression during metamorphosis were based on such processes as macrophage infiltration, lysosomal expansion or activation of lytic enzymes (Weber 1969, Tata 1994, Yoshizato 1996). However, lysosomal activators fail to activate directly these enzymes (proteases, nucleases, phosphatases) in their latent forms and also fail to induce tissue regression characteristic of metamorphosis. This raised the possibility that the hormonal elevation of lytic enzyme activity in larval tissue programmed for regression was caused by a selective enhancement of the synthesis of some or all of them. Studies on induction of regression in organ cultures of tadpole tails showed that $T_3$ simultaneously augmented the amount of several lytic enzymes and the protein and RNA synthesizing activity of the tadpole tails. The use of inhibitors of RNA and protein synthesis demonstrated the requirement of newly synthesized RNA and protein to initiate cell death during metamorphosis and other postembryonic developmental processes (Weber 1965). Later work from Brown's laboratory, based on recombinant DNA technology, made it possible to define the extent and nature of modulation by TH of expression of different sets of genes during tissue regression (Wang & Brown 1991, 1993).

In the mid-1960s some laboratories were able to maintain *Xenopus* and *Rana* tadpole tails in organ cultures for sufficiently long periods such that it was possible to induce tissue regression by the simple addition of TH to the culture medium (Tata 1966, Weber 1965). It was thus possible to induce with the hormone several lytic enzymes such as cathepsins, RNases and DNases which were accompanied by

regression of the cultured tails. Culturing also had the advantage that it was possible to simultaneously determine if the cell loss was accompanied by changes in protein and RNA synthesizing activities of the regressing tissue. The increase in lytic enzyme activity and tissue regression following the addition of $T_3$ to organ cultures of *Xenopus* tadpole tails was accompanied by a burst of RNA and protein synthesizing activity. This finding raised two important questions. Is the enhanced protein synthetic activity necessary for cell death? Are any new proteins synthesized at the onset of tissue loss and, if so, what is their nature? When inhibitors of RNA and protein synthesis, such as actinomycin D, puromycin and cycloheximide, were added to organ cultures of tadpole tails, along with or after $T_3$, a paradoxical result was obtained (Tata 1966, Weber 1965) . These cytotoxic agents, which normally kill cells, were found to protect cells programmed to die during postembryonic development. When the kinetics of tail regression were measured (figure 2), actinomycin D not only blocked tail regression when added along with $T_3$ at the beginning of the culture, but also when administered after $T_3$ had initiated regression (DNA measurements show that measuring tail length is a good index of cell loss). Thus, ongoing RNA and protein synthesis is necessary for programmed cell death (PCD) to be initiated as well as for it to continue.

As to the nature of any new proteins whose *de novo* or enhanced synthesis is essential for developmentally programmed cell death, it is only when recombinant DNA techniques, such as subtractive hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) were applied that it became possible to obtain some idea about the qualitative and quantitative aspects of new genes expressed during PCD in amphibian metamorphosis. In our laboratory we investigated the first possibility that the survival genes may be differentially regulated in tissues programmed for growth and regression during *Xenopus* metamorphosis (Cruz-Reyes & Tata 1995). For this purpose *Xenopus* bcl-2 like genes were cloned and two members of this family (xR11 and xR1), closely related to bcl-$X_L$, were studied in detail, as it is considered to be a major gene conferring survival function on cells (Korsmeyer 1995). Both xR11 and

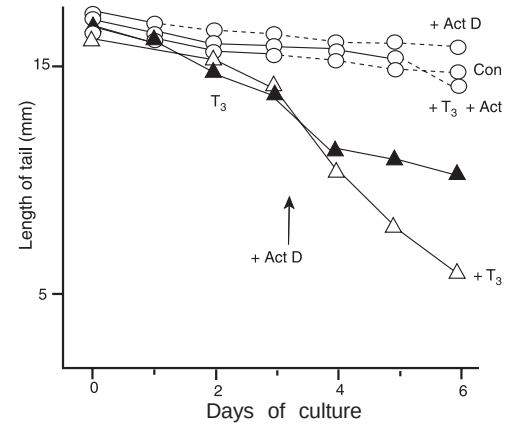


*Figure 2* Inhibition of RNA synthesis by actinomycin D (Act D) blocks $T_3$-induced regression of *Xenopus* tadpole tails in organ culture. Kinetics of *Xenopus* tadpole tail regression in culture following the addition of $T_3$ ($5x10^{-8}$ M) at the beginning of culture period (day O) and actinomycin D (5 µg/ml.) added at day O or day 3. The length of the tail is a good index of tissue regression and corresponds to the loss of total DNA during culture (Adapted from Tata 1966).

xR1 exhibited all the criteria of protecting heterologous cells against the apoptotic action of cytotoxic agents, such as staurosporine and cycloheximide, or cell death induction by c-*myc*. However, both xR1 and xR11 continued to be expressed in regressing as well as non-regressing tissues during natural and TH-induced metamorphosis. XR11 is indeed upregulated in the brain during metamorphosis. It is therefore most significant that in a recent study overexpression of xR11 in transgenic *Xenopus* the survival of Mauthner's and Rohon-Beard neurones in froglets that had just completed natural metamorphosis. Normally, these neurones undergo total or partial regression during natural or precociously hormone-induced metamorphosis (Coen et al. 2001). In Demeneix's laboratory *bax*, a related member of the *bcl*-2 family and a well established cell death effector in many organisms and cells, has also been shown to be involved in TH-mediated regression of *Xenopus* tail *in vivo* (Sachs et al. 1997).

Another major tissue undergoing extensive cell death during amphibian metamorphosis is the gut or small intestine. In *Xenopus*, nearly 90% of the tadpole's small intestinal epithelium is lost following the onset of metamorphosis (McAvoy & Dixon 1977, Hourdry & Dauca 1977). But unlike

tissues undergoing total regression, such as the tail and gills, the remaining 10% of the cells undergo rapid differentiation and multiplication to generate the adult digestive tract, also under the control of thyroid hormone. Ishizuya-Oka and her colleagues have carried out detailed studies on the regression and remodelling of the amphibian intestine during metamorphosis (Ishizuya-Oka & Shimozawa 1994, Ishizuya-Oka 1996, Ishizuya-Oka et al. 1996). By combining detailed analysis of ultrastructure and immunocytochemistry, these investigators have been able to show that the major target of $T_3$ is the connective tissue lying between the very simple larval intestinal epithelium and basement membrane. The hormone is thought to induce metalloproteinases which would act on the basement membrane and thus facilitate cell-ECM interaction. *In situ* hybridization has allowed the localization of the transcripts of one of the metalloproteinases, stromelysin-3, which is transiently expressed in the connective tissue during metamorphosis (Ishizuya-Oka et al. 1996). The cell-extracellular matrix (ECM) interactions induce PCD of the larval epithelium, as a result of which multiple differentiative steps lead to the formation of the adult intestine, characterized by a substantial thickening of the basement membrane and the formation of a new, highly convoluted intestinal epithelium. It has been suggested from co-culture experiments with different regions of the larval intestine have established that the death of larval epithelial cells may be necessary for differentiation and development of the adult intestine(Ishizuya-Oka & Shimozawa 1994, Ishizuya-Oka et al. 1996). Other hormones are also known to potentiate or inhibit TH-induced PCD. For example, exogenous glucocorticoids enhance $T_3$-induced regression of tadpole tail in organ culture while prolactin (PRL) suppresses this process (Tata et al. 1991). These effects are paralleled by the up- or down-regulation of the expression of TR and RXR (Iwamuro & Tata 1995). How these receptor mRNA changes correlate with $T_3$-induced PCD was demonstrated by an experiment in which the synthetic glucocorticoid dexamethasone (Dex) did not induce apoptosis on its own but speeded up tail regression induced by $T_3$. PRL slowed down considerably the Dexaccelerated rate of cell death as it did with $T_3$ alone. These responses were paralleled by the effects of Dex and PRL on the autoinduction of TRβ mRNA and the expression of genes encoding cell death effectors.

### N*uclear Receptors and Transcription*

The receptor is the key element of all cellular signalling mechanisms. There are two major classes of receptors for extracellular signals, such as hormones, growth factors, vitamins and neurotransmitters, which are distinguished according to their chemical nature and cellular localisation. A considerable literature has accumulated over the last decade about the molecular, cellular and structural biology of hormone receptors and the reader is referred to some excellent reviews on this topic (Mangelsdorf et al. 1995, Chambon 1995, Parker 1996, Cohen & Frame 2001, Laudet & Gronemeyer 2002, Brivanlou & Darnell. 2002, Tata 1998, 2002, Pierce et al. 2002). One class comprises receptors that are located in the cell membrane and are the cellular homologues of the oncogene erb B (c-*erb* B) whose ligands are peptide hormones and growth factors, such as insulin, growth hormone, epidermal growth factor (EGF), as well as neurotransmitters. They are characterised by their transmembrane localisation, signal recognition domain on the cell surface and an intracellular domain often linked to adenyl cyclase and G-proteins and the signal is often transduced to intracellular targets via a cascade of protein phosphorylation and dephosphorylation mechanisms.

The other class comprises nuclear receptors whose well-known ligands are steroids, thyroid hormone, retinoic acid and vitamin D. About twenty nuclear receptors have been characterized as cellular homologues of the oncogene erbA (C-*erb* A) often tightly bound chromatin proteins and function as ligand-activated transcription factors (Mangelsdorf et al. 1995, Wolffe 1998, Laudet & Gronemeyer 2002, Tata 2002). There are two subgroups of nuclear receptors: a) those that form complexes with heat-shock proteins and can activate transcription of target genes as monomers or homodimers; receptors for steroid hormones such as oestrogen, glucocorticoids and testosterone belong to this group; b) receptors for TH, retinoic acid, peroxisome proliferator activators and

vitamin D constitute the other sub-group of closely related nuclear receptors; these are characterized by the fact that they form heterodimers with a member of the same sub-group of nuclear receptors, namely retinod X receptor (RXR).

How is the high degree of target gene specificity for a given hormone and its receptor achieved? The answer lies in the highly precise spacing of nucleotide repeats in the hormone response element (HRE) of the promoter of the target gene and the DNA-binding domain (DBD) of the receptor which recognises it. A consensus hexanucleotide sequence, usually present as a pair, is the most common feature of all the known HREs but the sequence of each hexad and the relative position of the two hexads exhibit considerable variability to generate the high degree of specificity of interaction between the receptor and its target gene. Thus, the HREs recognised by these receptors (TR, RAR, RXR, VDR, PPAR) and EcR all share the same AGGTCA hexad and are organised as direct repeats (DRs) separated by one to five nucleotides (see figure 3). This arrangement of HREs is termed as the "1-2-3-4-5 rule" by Evans and colleagues explains the fine discrimination of target genes by the heterodimers formed by each of these receptors with RXR. The partners of RXR shown in figure 3 are distinguished by the number of nucleotides separating the two half-sites of the direct repeats of the HREs (Mangelsdorf et al. 1995) . This fine discrimination, which confers an extraordinary hormonal specificity, is surely a most remarkable example of selective transcriptional regulation. Dramatic confirmation of the above biochemical findings regarding the heterodimerisation andthe interaction between DBD of nuclear receptors and their cognate HREs has been provided by x-ray crystal structure analysis (Rastinejad et al. 1995)

Since nuclear hormone receptors are ligand-activated transcription factors, it is not surprising that an ever-increasing number of non-receptor transcription factors are found to interact with nuclear receptors and that these complexes may function synergistically or in a mutually antagonistic manner. Thus, large protein molecules termed CBP (CREB binding protein) and p300 have been shown to form bridges between nuclear hormone receptors and other

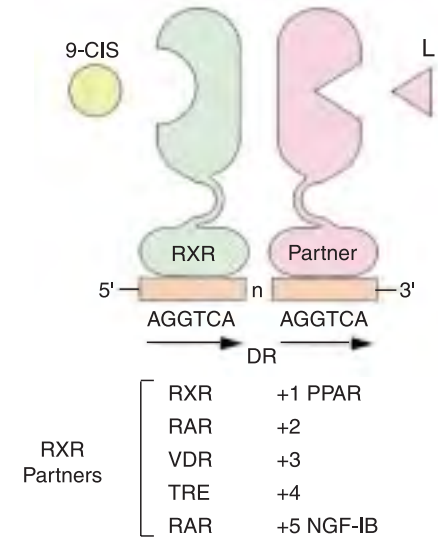


*Figure 3* The 1-2-3-4-5 rule. Non-steroidal nuclear receptors that function as heterodimers with RXR to recognize the direct repeat (DR) of the hexad AGGTCA separated by 1-5 nucleotides (n). RXR can form homodimers and NGF-1B is an orphan receptor. RXR, retinoid X receptor; *L*, ligand (9-cis retinoic acid for RXR); *PPAR*, peroxisome proliferator-activated receptor; *RAR*, retinoic acid receptor; *VDR*, vitamin D receptor; *TR*, thyroid hormone receptor; *NGF-1B*, nuclear-growth factor-inducible receptor. See Mangelsdorf et al. 1995 for a detailed account of RXR heterodimers.

transcription factors. This conclusion was arrived at by the unexpected observation that nuclear receptors inhibit the activity of the non-receptor transcription factor AP-1 by competing for limited amounts of CBP/p300 normally present in cells. Other important elements of the complex are the p160 nuclear receptor co-activator and the 270 kDa nuclear receptor co-repressor (N-CoR), which have been purified and their functions tested. A structural and thermodynamic analysis of the interaction domains of CBP and the p160 co-activators of TR and RAR revealed a mechanism of mutual synergistic folding whereby the co-activators recruit CBP/p300 to facilitate the transmission of the hormonal signal to the transcriptional machinery (Chakravarti et al. 1996, Demarest et al. 2002) Thus the CBP/p300 complex serves to integrate multiple signalling pathways in the cell nucleus. A number of transcriptional co-activators have now been characterised in mammals and similar work extended to amphibia in future will prove to be valuable. For example, Amano et al. (2002) have

recently shown that the *Xenopus* homologue of human co-activator Trip7 interacts with TR in a chromatin-dependent manner and is directly upregulated during metamorphosis.The functions of many transcription factors and nuclear receptors involved in such integration are themselves regulated by protein phosphorylation which would serve to set up a network linking membrane and nuclear receptor signal transduction pathway (Brivanlou & Darnell 2002).

Complexities of transcriptional regulation, the higher order of organisation of genes within the nucleus and the growing number of cross-interactions among regulatory factors have focused attention on the important role of chromatin structure in hormonal regulation of gene expression. Studies from Beato's laboratory have provided evidence that the function of glucocorticoid receptor in regulating the target gene promoter is determined by the manner in which it itself is organised within the chromatin structure (Beato et al. 1996) . According to a simple model presented by them, the HREs are highly organised in phased nucleosomes and the binding of the hormone to its nuclear receptor causes an alteration in the chromatin structure such that it will induce the binding of non-receptor transcription factors, such as NGF-1 and OTF-1, and thus allow the transcription of the hormone-regulated gene promoter. Similarly Wong et al. (1995) have suggested that both the silencing and activation of the *Xenopus* TRβ gene is determined by processes controlling nucleosome assembly. The conclusions of such chromatin studies have largely been inferred indirectly from techniques such as cross-linking to determine points of contact between DNA and protein (Wolffe 1998). Clearly, more direct information of the spatial organisation and mobility of receptors is needed before we can draw precise conclusions as to the role played by chromatin rearrangements within the nucleus *in vivo*. The recent development of the technique of chromatin immunoprecipitation is a promising step in this direction. It is therefore significant that Sachs & Shi (2002) have recently effectively exploited this technique to follow targeted chromatin binding and histone acetylation in vivo by TR during amphibian development.

*Regulation of Thyroid Hormone Receptor Genes during Metamorphosis*

There are two thyroid hormone receptor genes, termed TRα and β, in all vertebrates. While differences in the structure and properties between of the two TRs, such as ligand- and DNA-binding, are reasonably well understood for mammalian receptors (Laudet & Gronemeyer 2002) these are less well characterised for amphibian TRs. What is clear from studies on metamorphosis is that the expression in *Xenopus* tadpoles is under developmental control. Very small amounts of both TR transcripts can be detected in unfertilized eggs and early embryos. A substantial increase, particularly of TRα mRNA, occurs at around stage 44 which, quite significantly, is when the *Xenopus* tadpole first exhibits competence to respond to exogenous thyroid hormone (*see* Tata 1968). At this stage of development several tissues which are programmed to undergo major changes later during metamorphosis show high concentrations of TR mRNA, such as brain, liver, limb buds, small intestine and tail, as seen from both biochemical and *in situ* hybridisation analyses (Yaoita & Brown 1990, Kawahara et al. 1991). After stage 54 and until the completion of metamorphosis there is good correlation between the accumulation of TR transcripts and the circulating level of thyroid hormone in *Xenopus* tadpoles. As seen in figure 4, the relative amounts of TRα and β mRNAs vary according to the region of the tadpole, TRβ being more strongly expre ssed in the head region, and also according to the progression of metamorphosis after stage 54. This finding is in contrast to those reported by other workers who are unable to detect any TR beta transcripts in premetamorphic tadpoles (Yaoita & Brown 1990, Shi et al. 1996).

The correlation between the increase in TR mRNA and circulating thyroid hormone raised the question as to whether or not exogenous TH would precociously upregulate TR gene expression in pre-stage 54 tadpoles. Several experimental studies from the laboratories of Brown, Shi and Tata, based on Northern blotting, RNase protection assays and *in situ* hybridization of RNA from various *Xenopus* tadpole tissues all established that indeed $T_3$ causes a substantial autoinduction of TR mRNA (Tata 1996, Kawahara et al. 1991, Shi et al. 1996, Rabelo et al. 1994).

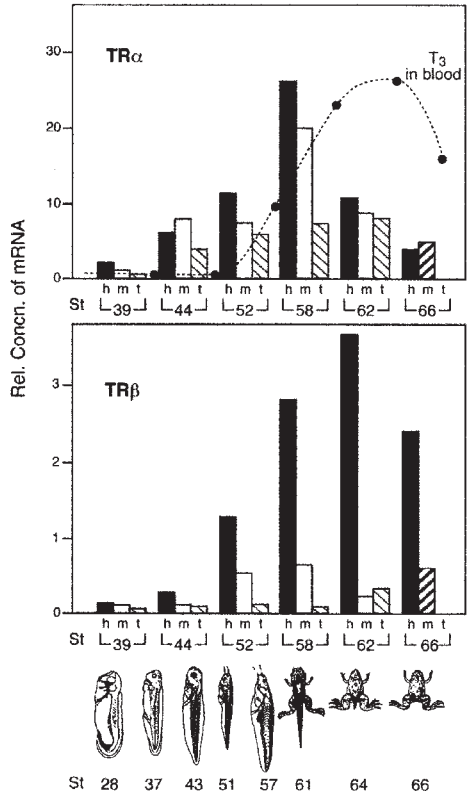


*Figure 4* Developmental regulation of *Xenopus* TRα and β gene expression during metamorphosis.

RNA was extracted from head (h), middle (m) and tail (t) regions of tadpoles at different stages (St) before (39, 44, 52), during (58, 62) and after (66) natural metamorphosis. Broken line denotes the levels of circulating $T_3$ during this developmental period. TR mRNAα accumulates in all tissues, and at all stages, to higher levels than does TRβ mRNA; note the 10-fold difference in scale for their relative amounts. Other details in Kawahara et al. 1991.

A similar upregulation of TR mRNA has been observed in *Rana catesbeiana* tadpoles (Atkinson et al. 1996). Some results obtained with *in situ* hybridization are illustrated in figure 5. Enhanced TR signals can be easily discerned in brain, intestine, and liver of pre-metamorphic (stage 52) *Xenopus* tadpoles exposed to low doses of $T_3$ for 4 days. A similar increased accumulation of TR mRNA is also visible in stage 53 tadpoles hind limb buds following $T_3$ treatment for 5 days. Note that in stage 53 tadpoles 5 day $T_3$ treatment has produced considerable growth of the hind limb bud accompanying the enhanced accumulation of TR mRNA (compare figure 5g and h). The same phenomenon of autoinduction of TR mRNA is observed for *Xenopus* tadpole tails *in vivo* and in organ culture. Note that these different tissues exhibit the initiation of different programmes of structural and biochemical modifications, *de novo* morphogenesis and cell death during both normal and hormone-induced metamorphosis.

It is worth considering some important features of TR autoinduction to assess its significance in the action of TH in regulating metamorphosis. (1) The extent of autoinduction of TR is dependent on the developmental stage of the tadpole. $T_3$ is ineffective at the very early developmental stages, the auotoinducibility increasing with development, the highest sensitivity being reached as metamorphosis progresses towards its climax. (2) The magnitude and kinetics of TR mRNA upregulation varies from tissue to tissue, cell type to cell type and in different regions of the tadpole. (3) Autoinduction, which is not restricted to amphibia, is more marked for TRβ mRNA than for TRα. In all organisms, including amphibia, TRα transcripts are generally significantly more abundant than those of TRβ.

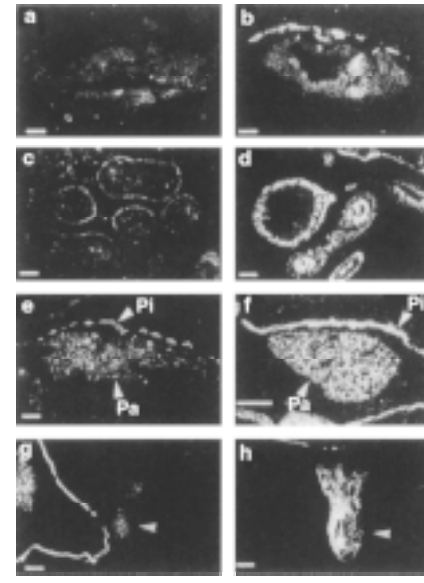


*Figure 5* Upregulation by $T_3$ of TR mRNA in brain (a,b), small intestine (c.d), liver (e,f) and hind limb buds (g,h) of premetamorphic (stage 52/53) *Xenopus* tadpoles. The illustrations are dark-field imaging of localization by in situ hybridization with antisense *Xenopus* TR cRNA . Saggital sections were cut from different tissues of control (a,c,e,g) or treated ($10^{-9}$M $T_3$ for 4 days; b,d,f, or 5 days for h) tadpoles. Sense probe gave virtually no signal and those images are therefore not shown. Arrows in g and h show hind limb buds. Arrows in e and f indicate parenchymal liver cells (Pa) and an artefact produced by pigmentation surrounding these cells (Pi). Bars are 100 μm. For more details see Rabelo et al. 1994.

With regard to kinetics of TR autoinduction, the relative amount of TRα mRNA in whole stage 52 *Xenopus* tadpoles is increased 2 to 4-fold 48 h after $T_3$, whereas TRβ transcripts increased 20 to 50-fold. What is particularly important is that an upregulation of TRβ mRNA can be seen as early as 4 h after the exposure of these pre-metamorphic tadpoles to exogenous $T_3$, which is among the most rapid biochemical responses of *Xenopus* tadpoles. Furthermore, TRβ is a direct-response gene which, together with the rapid response, raises the possibility that upregulation of TR is a requirement for gene programming during hormonal induction of metamorphosis. Autoinduction of TRα and β mRNAs can also be reproduced in permanent, non-transformed, *Xenopus* cell lines. Cultures of XTC-2 and XL-177 cells are particularly responsive to $T_3$ (Machuca & Tata 1992, Kanamori & Brown 1993), the kinetics of upregulation of the two receptor transcripts in these cell lines being very similar to those induced in whole tadpoles.

In contrast to the numerous studies on transcription of nuclear receptor genes there are very few reports on tissue distribution or developmental and hormonal regulation of expression of receptor proteins. More specifically, as regards amphibian metamorphosis, only two publications deal with TRα and β-proteins during *Xenopus* development and metamorphosis. Using polyclonal antibodies to detect *Xenopus* TRs in embryo or tissue extracts, Elicieri and Brown (1994) were able to find TRα, but not TRβ, in unfertilized eggs, embryos and all stages of tadpoles before and during metamorphosis. These investigators could only detect TRβ at stages when endogenous TH was secreted, i.e. at the onset of natural metamorphosis, or if exogenous $T_3$ was administered to pre-metamorphic tadpoles. The amount of two proteins was found to increase roughly as a function of the enhanced accumulation of their respective mRNAs, which led them to suggest that TRβ was induced by the liganded TRα. However, when Fairclough and Tata (1997) used specific monoclonal antibodies to detect the two receptor isoforms immunocytochemically, it was possible to demonstrate the presence of both TRα and β proteins in the nuclei of all tissues examined from tadpoles before and during natural metamorphosis. Administration of $T_3$ nevertheless augmented the amount of both proteins in tadpole liver and small intestine. The first tissue undergoes extensive gene switching and functional maturation whereas the latter loses about 90% of its cells followed by rapid morphogenesis. It was not possible to show immunocytochemically an exact equivalence between TR mRNA and protein content, nor was it possible to show a very marked effect of TH on the upregulation of both TRs in the brain. The reasons for this partial discrepancy between the results obtained with quantitative immunoprecipitation (Elicieri & Brown 1994) and immunocytochemistry (Fairclough & Tata 1997)are not clear, but one has to consider the different sensititvities of the two techniques when used quantitatively.

***Mechanism of Autoinduction of TR***

The simplest mechanism to explain the phenomenon of autoregulation of *Xenopus* TR would be a direct interaction between TR proteins and the promoters of the genes encoding them, providing the promoter contains one or more thyroid hormone responsive element (TRE). Since the expression of *Xenopus* TRβ-gene is modulated by $T_3$ to a greater extent than that of the α gene (see figure 4), it is significant that the promoter of the β gene has been cloned and characterized in greater detail (Shi et al. 1992, Machuca et al. 1995).

Two TREs of the more common DR+4 (direct repeat +4) type have been identified in 1.6 kb of the upstream sequence of the *Xenopus* TRβ gene. It is significant that an RXRE, the response element of TR's partner RXR, was also located near the TREs. Transfection of *Xenopus* XTC-2 and XL-2 cells, which express both TRα and β with CAT constructs of various TRβ promoter fragments, showed that both TREs responded to $T_3$. Interestingly, overexpression of unliganded TRα and β in these *Xenopus* cell lines caused a substantial suppression of basal transcriptional activity. This transcriptional repression by unliganded TRs has been well characterized in mammalian systems (Lazar 1993).Under the conditions of transcriptional suppression, the addition of $T_3$ to *Xenopus* cells, co-transfected with the full-length TRβ promoter, produces up to 20-fold enhancement of CAT activity, i.e. TR gene transciption (Machuca et al. 1995). Electrophoretic mobility shift assays (EMSA),

performed with the TRβ promoter fragments and recombinant *Xenopus* TRα and β supported the findings of transcriptional activities. Both TREs strongly interact with the receptors which could only be observed when recombinant *Xenopus* RXRα, β or γ were added together with TRα or β. These EMSAs clearly demonstrate that TR-RXR heterodimers (in any combination), but not TR monomers or homodimers, specifically interact with the DR+4 TREs of *Xenopus* TRβ gene promoter. While these studies do not rule out the participation of different response elements or other accessory factors (see Tata 1997, 1998) they strongly support the idea of a direct interaction between the thyroid hormone receptor and the promoter of its own gene as the most likely mechanism underlying *Xenopus* TR-autoinduction.

A direct approach based on TR gene 'knock-out', as has been achieved in mice (Forrest & Vennstrom 2000, O'Shea & Williams 2002), would provide strong evidence for the physiological significance of auto-upregulation of TR. But since homologous recombination has not yet been successfully achieved in amphibia, it is necessary to rely on indirect approaches. One such approach taken in our laboratory is based on the use of dominant negative TRs (Ulisse et al. 1996). Virtually all the mutations described involve deletion, frame-shift or addition in or around the ligand-binding domain of the β gene (Yen & Chin 1994). The mutant receptor fails to bind TH and will prevent the liganded normal wild-type receptor from interacting with TREs and activating transcription. Ulisse et al. (1996) exploited these naturally occurring and artificially generated human and *Xenopus* dominant-negative TRs to delve further into the mechanism of autoregulation of TR and its significance in $T_3$-induced metamorphosis (see figure 6). The human dominant-negative mutant TR and the synthetic *Xenopus* TRβ construct, homologous to a C-terminal human mutant receptor, abolished the ability of $T_3$ to upregulate endogenous or over-expressed wild-type *Xenopus* TR when these were transfected into *Xenopus* XTC-2 cells. The recombinant mutant TR proteins heterodimerized with *Xenopus* RXR to interact strongly with the DR+4 TREs present in *Xenopus* TRβ gene promoter. Ulisse et al. (1996) extended

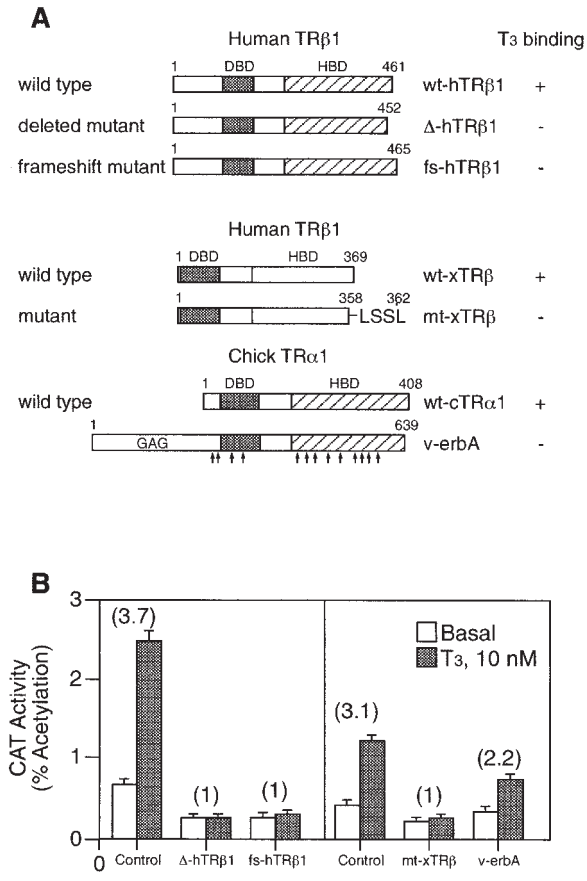


*Figure 6 A*. Organization and ligand binding properties of wild-type and mutant human, *Xenopus* and chicken TR and the viral oncogene v-*erb*A. One of the mutant human TRβs ( -hTRβ1) has a deletion of the last 9 amino acids, while the other (fs-hTRβ1) is a frameshift mutation caused by a 7-nucleotide duplication. The artificially mutated *Xenopus* TRβ (mt-xTRβ) has the last 11 amino acids of the wild type receptor (wt-xTRβ) replaced by 4 amino acids. The v-*erb*A oncogene shows N- and C-terminal deletions along with several internal mutations (arrows) compared with the wild-type chicken TRα1 (wt-cTRα1). The $T_3$ binding properties are denoted as + (normal) and - (no) binding. DBD and HBD denote DNA- and hormone-binding domains, respectively. The numbers refer to amino acid positions; *B*, Dominant-negative activity of mutant human and *Xenopus* TRβ and v-*erb*A in *Xenopus* XTC-2 cells. The cells were transfected with a wild-type *Xenopus* TRβ promoter with a CAT reporter (p[-1500/+87]xTRβ-CAT) construct with or without the TR mutants Δ-hTRβ1, fs-hTRB1, mt-xTRβ or v-*erb*A, as depicted in (*A*). CAT activity was measured and the values in parenthesis are fold-stimulation of activity when the cells were incubated with $T_3$. Note that all three mutant TRβs, but not v-*erb*A, cause a reduction of basal CAT activity. For details see Ulisse et al. 1996.

these experiments in *Xenopus* cell lines to co-inject dominant-negative and wild-type receptor constructs with TR TRE-CAT reporter into pre-metamorphic *Xenopus* tadpole tails. Culturing these tails in the presence of $T_3$ reproduced the repressor activity of dominant-negative mutants in vivo. This inhibition of upregulation of TRβ is relevant to the significance of auto-upregulation of TR. Two very recent studies from Brown's laboratory (Schreiber et al. 2002, Das et al. 2002) based on the expression of a dominant negative form of TR in transgenic *Xenopus* and another involving the use of Hammerhead ribozymes to inhibit TH-induced transcription in cultured *Xenopus* cells (Lim & Furlow 2002) have revealed new complexities in cell death and growth programmes as well as between TRα and β. Another precise approach to the question of establishing the nature of newly expressed genes would be to exploit the recently introduced technique of small RNA interference (RNAi) (Elbashir et al. 2001). Future studies along this line should provide a deeper insight into the mechanism of receptor autoinduction.

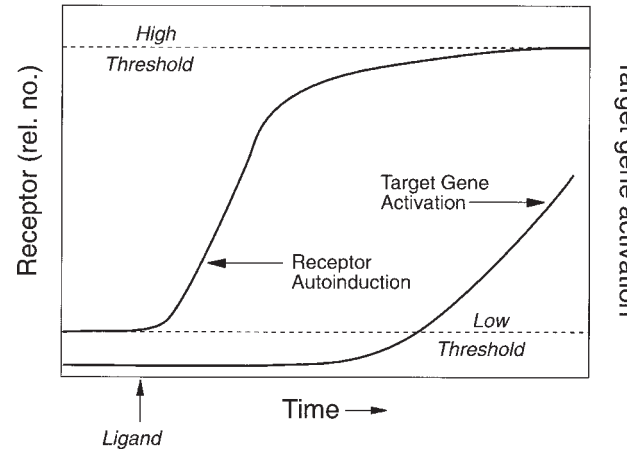


*Figure 7* A simplified dual-threshold model to explain the physiological significance of auto-upregulation of nuclear receptors in hormonally regulated developmental processes. According to this model a given receptor is constitutively expressed at a level too low to activate the target genes that specify a particular developmental function (lower threshold). Upon the release of the hormone the liganded receptor is capable of inducing its own receptor by virtue of a high affinity interaction with the promoter of the gene encoding the receptor gene. When the latter reaches a certain higher threshold level that it is now capable of activating the downstream target genes responsible for the phenotypic response.

**W***ider Significance of Autoregulation of TR during Metamorphosis*

The autoinduction phenomenon, described above, is not restricted to TR upregulated by TH during amphibian metamorphosis but is also seen with the expression of other nuclear receptors under the control of their own hormonal ligands (Tata 1998, 2000, 2002). What can be the significance of auto- and cross-induction of nuclear receptors? There is now good evidence to suggest that receptor up-regulation is linked to the biological activity of the ligand, at least where it concerns developmental action. A model (figure 7) can be proposed, whereby upregulation of a given receptor is a pre-requisite for the sequential activation of its target genes that specify its biological action. It predicts a double threshold of receptor numbers, the first for the autoinduction of the receptor and the second for the activation of target genes. It also implies that the gene encoding a given receptor is constitutively expressed to produce a very low level of functional receptor in the target tissue at very early stages of development, which indeed is the case for most growth and developmental hormones and growth factors. Upon the onset of secretion of the hormonal ligand, the low concentrations of the liganded receptor would only suffice to activate its own receptor gene, but not of its target genes which would require a significantly higher concentration of the hormone and receptor. Measurement of the relative affinities of interaction between the DNA-binding domain of the receptor and the response element in the promoters of the receptor and target genes would be a first step to validating this model. The above considerations raise the intriguing question of whether the target cell's response to the hormone is determined by receptor abundance or occupancy. While it is not possible to obtain direct experimental evidence *in vivo* for one or the other , the two mechanisms are not mutually exclusive, so that the answer is unlikely to be "either or". Another intriguing question arises as to what are the factors that maintain the low levels of the constitutively expressed receptor prior to the onset of the developmental process which is accompanied by the release of the hormone. Of course, this is not the only problem to be resolved, but, as with so many other branches of life sciences, new technological tools will certainly lead to their resolution. Among

some useful considerations in addressing these questions are: a) a comparative approach receptors of amphibia in which metamorphosis is only partial, as in urodeles such as axolotls and newts (Just et al. 1981) or in anurans where metamorphic priod is greatly compressed, as in 'direct developing' frogs (Callery & Ellison 2000) applying recently established techniques such as transgenesis (Das et al. 2002, Schreiber et al. 2001) and the inhibition of specific gene expression with interference RNA (RNAi) and ribozymes (Lim & Furlow 2002). Meanwhile, it is clear that amphibian metamorphosis has served as a most fruitful model for advancing our knowledge of hormonal signalling and the regulation of postembryonic development.

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