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Review

Auto-regulation of thyroid hormone receptor genes during metamorphosis: roles in apoptosis and cell proliferation

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CONTENTS

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Introduction

Proper development and function of various organs in multicellular organisms are dependent upon the number and differentiation states of various cells within different tissues. It has long been established that cell proliferation and differentiation are key players in organogenesis and organ function. It is only until fairly recently that it has been accepted that cell elimination through programmed cell death plays crucial roles in maintaining cellular homeostasis in many developmental and pathological processes (Wyllie *et al.*, 1980; Schwartzman and Cidlowski, 1993; Jacobson *et al.*, 1997).

Amphibian metamorphosis represents one of the most dramatic postembryonic developmental processes where extensive cell elimination and proliferation participate in the proper formation of adult organs. This tadpole-to-frog transition systematically transforms essentially all tissues and organs in a tadpole (Dodd and Dodd, 1976; Gilbert and Frieden, 1981). However, different organs undergo vastly different transformations. The *de novo* development of adult organs such as the limb represents an extreme case where cell proliferation and differentiation play dominant roles. On the opposite end, the resorption of tadpole specific organs such as the tail involves mostly cell death. The vast majority of the tissues/organs are present both in tadpoles and frogs and undergo partial but drastic remodeling during metamorphosis.

One of the better studied organ remodeling processes is the intestinal transformation (Dauca and Hourdry, 1985; Shi and Ishizuya-Oka, 1996). The tadpole intestine is predominantly a single tubular layer of larval epithelial cells with little conntective tissue or muscles (see stages 51 to 55 in Fig. 1 for schematics of the intestinal cross-sections for Xenopus *laevis*. McAvoy and Dixon, 1977; Marshall and Dixon, 1978; Ishizuya-Oka and Shimozawa, 1987). This simple structure is replaced during metamorphosis by a multiply folded adult epithelium, which is surrounded by elaborate connective tissue and muscles (Fig. 1). This transformation in the gastrointestinal tract is accompanied by a change from being a herbivorous tadpole to a carnivorous frog (also see Smith-Gill and Carver, 1981; Yoshizato, 1989).

Abbreviations used in this paper: TR, thyroid hormone receptor; T3, thyroid hormone or 3, 3' 5-triiodiothyronine; RXR, 9-cis rentinoic acid receptor; TRE, thyroid hormone response element; CsA, cyclosporin A.

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While extensive and complex, the entire metamorphic process is controlled by thyroid hormone (T_3) (Dodd and Dodd, 1976; Gilbert and Frieden, 1981; Kikuyama *et al.*, 1993). Thus, blocking the synthesis of endogenous T_3 inhibits metamorphosis while adding exogenous T_3 to premetamorphic tadpoles (e.g., before stage 55 for *Xenopus laevis*, Fig. 1) induces precocious transformations. Furthermore, the control by T_3 appears to be organ autonomous as individually dissected tadpole organs such as the limb, tail, and intestine, can undergo metamorphic transformations when cultured *in vitro* in the presence of T_3 (Dodd and Dodd, 1976; Ishizuya-Oka and Shimozawa, 1991; Tata *et al.*, 1991).

The effects of T_3 are believed to be mediated by thyroid hormone receptors (TRs), which are nuclearly localized high affinity T_3 binding proteins (Sap *et al.*, 1986; Weinberger *et al.*, 1986). TRs can regulate transcription of target genes in a T_3-dependent manner, thus affecting cellular events. In this article, we will review some recent findings on the expression, especially the autoregulation, of the TR genes during amphibian metamorphosis and the evidence pointing toward a role of TRs in both cell death and proliferation in tissue remodeling. While the bulk of the data reviewed here is based on studies in *Xenopus laevis*, the conclusions are believed to be generally applicable to other amphibians.

Apoptosis and cell proliferation during metamorphosis

The remodeling of various tadpole organs during metamorphosis involves an intricate control of cell proliferation and elimination (Dodd and Dodd, 1976; Gilbert and Frieden, 1981). The development of adult organs requires first the proliferation and then differentiation of adult cells. This is especially true for adult specific organs such as the limb. Even in such cases, specific cell death, e.g., in the interdigital region of the limb, is likely to play important roles for proper morphogenesis. On the other hand, cell proliferation may also be an important factor even in organs undergoing complete resorption. This is in part due to the fact that different cell types of the resorbing organs, such as the tail, are resorbed at distinct stages to coordinate the resorption of the organs and at same time to maintain certain physiological functions of the organs that are required before the completion of metamorphosis. It is commonly accepted that cell proliferation and differentiation are genetically controlled events critical for adult tissue development. However, the evidence showing that larval cell removal is an active, hormonally controlled cellular event has been accumulating more slowly. The following section reviews some of the findings demonstrating that larval cell removal is through programmed cell death with apoptotic morphology.

Apoptotic cell elimination in vivo

Cells undergoing programmed cell death are often accompanied by a series of well defined morphological changes (Wyllie *et al.*, 1980). These include blebbing of the cell membrane, chromatin condensation, fragmentation of the nucleus as well as the cell itself to form the so-called apoptotic bodies containing condensed chromatin fragments and/or cellular organelles encircled by membrane. Due to the efficient and fast removal of apoptotic cells and apoptotic bodies by neighboring cells, especially macrophages, programmed cell death is often difficult to observe *in vivo*. On the other hand, tadpole tail resorption represents a case where cell death takes place at its extreme. Thus, using an electron microscope and the cellular morphological criteria, Kerr *et al.* (1974) observed more than 20 years ago that tail muscle cells undergo apoptosis during metamorphosis. Subsequently, it has also been found that the degeneration of larval intestinal epithelium also involves apoptosis (Fig. 1; Ishizuya-Oka and Shimozawa, 1992b). Moreover, the resulting apoptotic bodies are often engulfed by macrophages that migrate into the larval epithelium after crossing the basement membrane separating the epithelium and the connective tissue (Ishizuya-Oka and Shimozawa, 1992b; Shi and Ishizuya-Oka, 1996).

T_3-induced apoptosis in vitro

The observation of apoptotic cells *in vivo* suggests that metamorphic cell death is an active cellular response, directly or indirectly, to T_3. This apoptotic response to T_3 is apparently organ autonomous. Thus, when dissected intestinal fragments are cultured *in vitro* in the presence of T_3, they undergo similar changes, i.e., the degeneration of larval epithelium through apoptosis and development of the connective tissue and adult epithelium (Ishizuya-Oka and Shimozawa, 1991,1992a,b; Ishizuya-Oka and Ueda, 1996). Similarly, the tadpole tail can also be induced by T_3 to resorb *in vitro* in organ cultures and this T_3-dependent resorption requires new protein and RNA synthesis (Tata, 1966; Tata *et al.*, 1991), consistent with the fact that the process is through programmed cell death.

Two studies on the tadpole tail have suggested that adult-type non-T leukocytes may participate in the specific elimination of tadpole tail tissues (Izutsu and Yoshizato, 1993; Isutsu *et al.*, 1996). On the other hand, by culturing dissociated cells from tadpole tail, Yaoita and Nakajima (1997) have established a stable cell line from tail muscle cells. The cell line can undergo apoptosis in response to T_3, suggesting that at least some of the larval cells can respond directly to T_3. In support of this, Ishizuya-Oka and Shimozawa (1992a) have shown that while the intestinal connective tissue is required for the development of adult intestinal epithelium *in vitro*, the intestinal larval epithelium undergoes apoptosis even when cultured alone in the presence of T_3.

More recently, we have isolated the intestinal epithelial cells from premetamorphic tadpoles of *Xenopus laevis* (Su *et al.*, 1997a). When cultured in the presence of T_3, these cells undergo cell death with apoptotic morphology and produce a nucleosomal-sized ladder of nuclear DNA fragments, typical of mammalian cell death processes (Su *et al.*, 1997a; Fig. 2A). This T_3-dependent cell death can be inhibited by many known inhibitors, such as inhibitors of ICE-like proteases and nucleases, of mammalian apoptosis (Su *et al.*, 1997b). Thus, the presence of immunosuppressants cyclosporin A (CsA), a known inhibitor of activation-induced T cell death (Shi *et al.*, 1989), during the T_3 treatment of these epithelial cells blocks the formation of the nucleosomal-sized DNA ladder (Fig. 2A). Furthermore, flow cytometry analysis has revealed that cells at different stages of cell cycle (i.e., with different DNA contents) can all undergo apoptosis in response to T_3 and CsA-inhibition of this T_3-dependent apoptosis is independent of cell cycle (Fig. 2B). Thus, the apoptosis of intestinal epithelial cells is a direct cellular response to T_3 and involves similar cell death effectors such as ICE-like

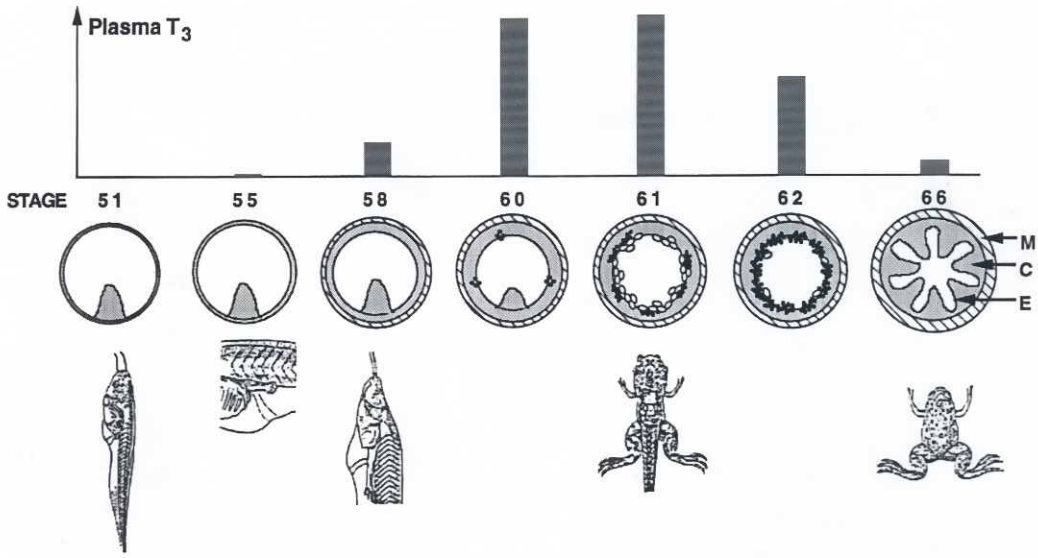

Fig. 1. Structural changes in the intestine during amphibian metamorphosis. *Schematic cross sections of the frog intestine are shown at different development stages according to Nieuwkoop and Faber (1956); McAvoy and Dixon, (1977); Kordylewski (1983); and Ishizuya-Oka and Shimozawa, (1987). During premetamorphosis (stages 51-55), the larval tadpole intestine consists of an epithelial layer with a single fold called the typhlosole. As metamorphosis proceeds to the climax at stages 60-63, the larval epithelium undergoes apoptosis (open circles) and is replaced by proliferating adult epithelium (filled circles). Towards the end of stage 62, apoptotic cells are localized in the tips of newly forming intestinal folds (not shown), although their number is small. Connective tissue starts to increase in thickness around stage 58 and muscle development takes place somewhat later. By the end of metamorphosis (stage 66), the frog intestine has many epithelial folds (E) along with well developed connective tissue (C) and muscle (M). Shown at the top are the relative levels of T_3 in the plasma (Leloup and Buscaglia, 1977).*

proteases as in mammalian cell death (Martin and Green, 1995; White, 1996).

T_3 regulation of *TR* genes during metamorphosis

As the presumed mediators of the causative effects of T_3 during amphibian metamorphosis, TRs have been a major focus of metamorphic research since the early days (Gilbert and Frieden, 1981; Galton, 1983; Gilbert *et al.*, 1996). The identification of TRs as high affinity T_3-binding proteins localized in the nucleus led to the suggestion that T_3 regulates metamorphosis by influencing genes expression (Gilbert and Frieden, 1981; Galton, 1983). This idea was supported when the avian and mammalian TRs were cloned and found to act as transcription factors (Sap *et al.*, 1986; Weinberger *et al.*, 1986; Evans, 1988; Green and Chambon, 1988). Subsequently, several laboratories have cloned one *TRα* and one *TRβ* gene from Ra*na catesbeiana*, and two *TRα* and two *TRβ* genes from *Xenopus laevis* (Brooks *et al.*, 1989; Yaoita *et al.*, 1990; Schneider and Galton, 1991; Helbing *et al.*, 1992).

TR gene expression is correlated with tissue remodeling

The cloning of amphibian *TR* genes has allowed the analysis of the expression of their mRNAs and proteins during development (Yaoita and Brown, 1990; Kawahara *et al.*, 1991; Schneider and Galton, 1991; Helbing *et al.*, 1992; Eliceiri and Brown, 1994; Fairclough and Tata, 1997). The regulation of the protein levels of a *TR* gene is generally in agreement with those of the TR mRNA. However, some discrepancies do exist and the studies on the protein expression are very limited (Eliceiri and Brown, 1994; Fairclough and Tata, 1997). Regardless, both the *TRα* and *TRβ* genes are expressed during metamorphosis of Rana catesb*eiana* and *Xenopus laevis*. Furthermore at least in X*enopus laevis*, the *TR* genes are all up-regulated by T_3 treatment of premetamorphic tadpoles (Yaoita and Brown, 1990; Kawahara *et al.*, 1991). In particular, the *Xenopus TRβ* genes have been shown to be directly regulated at the transcriptional level by T_3 through at least one thyroid hormone response element (TRE) in their promoters (Ranjan *et al.*, 1994; Machuca *et al.*, 1995). These results implicate that *TRs* auto-regulate their own expression to facilitate the drastic metamorphic changes needed within a short developmental period.

Supporting a critical role of *TRs* during metamorphosis is the strong temporal correlation of the TR mRNA levels with tissue specific transformations in *Xenopus laevis* (Wang and Brown, 1993; Shi *et al.*, 1994; Wong and Shi, 1995). Thus, in the hindlimb of *Xenopus laevis*, The *TR* mRNAs are expressed at higher levels during stages 54-58 but lower levels afterwards. Stages 54-58 correspond to the period when limb morphogenesis takes place while stages 58-66 are the stages of limb growth with little morphological changes (Nieuwkoop and Faber, 1956; Fig. 1). Similarly when the tail is being resorbed toward the end of metamorphosis (stages 62-66, Nieuwkoop and Faber, 1956; Fig. 1), the *TR* genes are highly expressed. Interestingly, in the intestine, the *TRα* mRNA levels do not change significantly during metamorphosis (Shi *et al.*, 1994; Wong and Shi, 1995). The *TRβ* genes, on the other hand, are highly up-regulated as the intestine remodels between stages 58-66 (Fig. 1). These correlations implicate that the temporal regulation of *TR* gene expression plays a role in determining when a specific tissue undergoes its metamorphic transformation.

Cell-type specific expression of TRβ genes correlates with apoptosis and cell proliferation in the Xenopus intestine

The tadpole intestine offers a unique opportunity to investigate the role of *TR* genes, especially the *TRβ* genes, during metamorphosis. As summarized above, the *TRβ* genes are direct T_3-response genes and have little expression before or after metamorphosis but are highly expressed during intestinal remodeling (Wong and Shi, 1995). Furthermore, the intestine consists of essentially three major types of tissues that are well-separated spatially and easily identifiable (Fig. 1). These tissues within the intestine undergo distinct metamorphic changes at different stages.

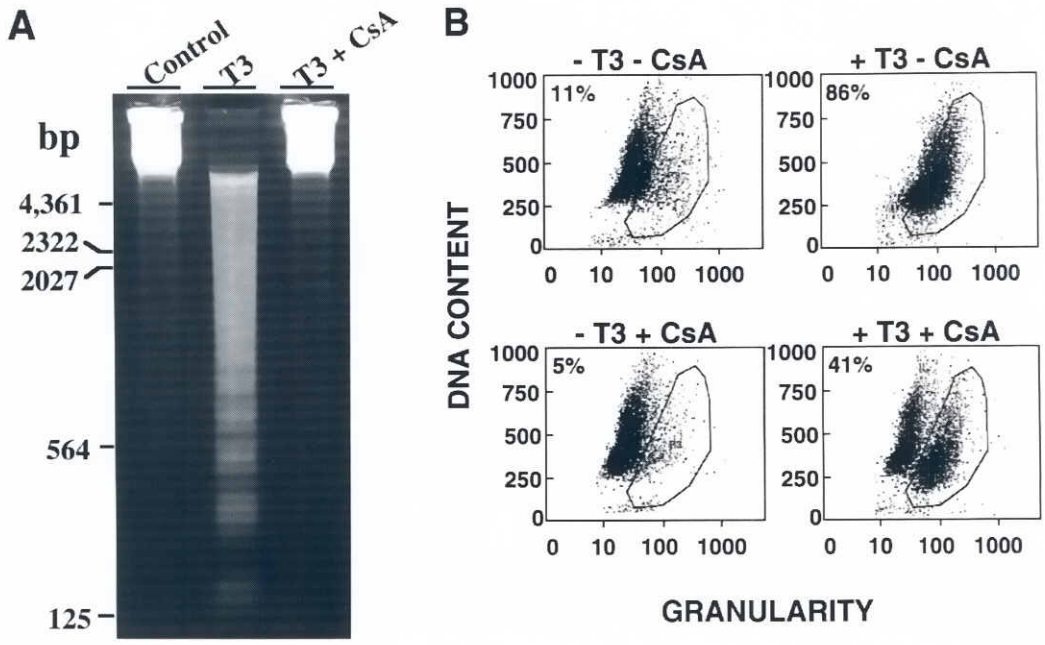

Fig. 2. Tadpole intestinal epithelial cells undergo apoptosis when cultured *in vitro* in the presence of T_3 (Su *et al.*, 1997a,b). (A) *T_3-treatment results in the formation of a nucleosomal-sized DNA ladder which can be inhibited by Cyclosporin A (CsA), a known inhibitor of activation-induced T cell death (Shi et al., 1989). The epithelial cells were treated with 0 or 100 nM T_3 and/or 600 ng/ml CsA for one day. The genomic DNA was then isolated and analyzed on an agarose gel.* **(B)** *Flow cytometry analysis indicates that epithelial cells at different stages of cell cycle undergo apoptosis in response to T_3. The epithelial cells were cultured in the presence or absence of 100 nM T_3 and/or 600 ng/ml CsA for three days. The cell were then analyzed by flow cytometry. Although the exact boundary between live and apoptotic cells (encircled) was hard to be fixed, the results clearly showed that cells with all different DNA contents or at different cell cycle stages (G2 at the top and G1 at the bottom) were present in the apoptotic region (as reflected by the increased cellular granularity). The percentage of the cells in the apoptotic region is indicated for each culturing condition. The results show that CsA inhibits apoptosis independently of cell cycle.*

Thus, a simple analysis of the *TRβ* gene expression in different cell types during metamorphosis may provide important clues on the role of *TRβ* in cell death or proliferation and differentiation. The larval epithelial is the first one to change and its apoptotic degeneration takes place around stages 60-62 (McAvoy and Dixon, 1977; Ishizuya-Oka and Ueda, 1996; Shi and Ishizuya-Oka, 1996). The adult epithelial development begins around stage 60 when proliferating adult epithelial islets are first identifiable (Fig. 1; McAvoy and Dixon, 1977; Ishizuya-Oka and Shimozawa, 1987). Active cell proliferation takes places around stages 61-62 and subsequently, the epithelial cells differentiate to form the multiply folded adult epithelium (Fig. 1; McAvoy and Dixon, 1977). The connective tissue, on the other hand, actively proliferate around stages 58-62 and their differentiation takes place toward the end of metamorphosis (Ishizuya-Oka and Shimozawa, 1987; Shi and Ishizuya-Oka, 1996). Finally, the muscles develop somewhat later than the connective tissue and the adult epithelium with the outer longitudinal muscle layer being the last one to attain its adult form among the intestinal tissues among the intestinal tissues (Kordylewski, 1983).

*In situ hy*bridization using a TRβ antisense RNA probe indeed reveals a strong correlation of *TRβ* gene expression with cell type specific changes in the *Xenopus* intestine (Shi and Ishizuya-Oka, 1997). The *TRβ* mRNAs are absent or at very low levels prior to stage 55. They are first up-regulated in the larval epithelium, to the maximal levels by stages 59-60, which is the onset or immediately prior to larval epithelial cell death. Interestingly, the mRNA levels are down-regulated as the cells undergo apoptosis (stages 60-62). The up-regulation of the *TRβ* mRNAs occurs around stage 60-62 in the adult epithelium, connective tissue, and muscles. The genes are down-regulated again in a sequential order in the adult epithelium, connective tissue, and muscles as their cells differentiate. In particular, the down-regulation occurs last in the longitudinal muscle, which is also the last tissue to attain its adult form. Thus, *TRβ* appears to be involved in the early stages of apoptosis and adult cell proliferation but is not required or only required at very low levels for differentiated adult cells.

Function of *TRs* in frog development

Mechanism of TR action

TRs are ligand-dependent transcription factors belonging to the superfamily of nuclear hormone receptors (Lazar, 1993; Tsai and O'Malley, 1994; Yen and Chin, 1994; Mangelsdorf *et al.*, 1995). A DNA binding domain is located within the N-terminal half of the protein and the T_3-binding domain in the C-terminal half. A transcriptional activation domain is present at the very C-terminal end of the receptor.

Extensive *in vitro* biochemical and tissue culture transfection studies have strongly implicated that *TRs* most likely function as heterodimers formed with RXRs (9-cis retinoic acid receptor) (Forman and Samuels, 1990; Yu *et al.*, 1991; Heyman *et al.*, 1992; Leid *et al.*, 1992; Marks *et al.*, 1992; Zhang *et al.*, 1992; Tsai and O'Malley, 1994; Yen and Chin, 1994). In the presence of T_3, TR/RXR heterodimers can activate the transcription of their target genes. However, in the absence of the ligand, TR/RXR can repress the target promoters. While the exact mechanisms for the repression and activation are unknown at present, they are believed to involve TR-interacting corepressors and coactivators, respectively (Fig. 3). Many potential cofactors have been isolated (Halachmi *et*

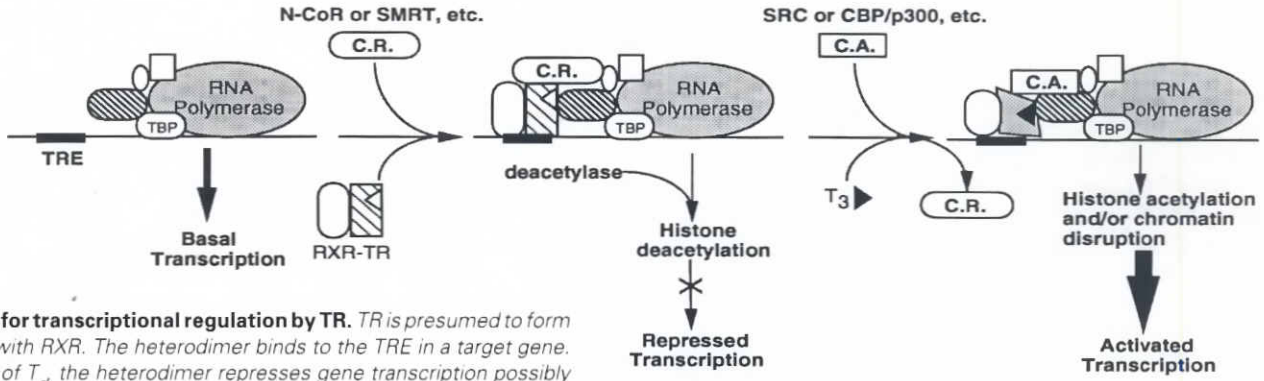

Fig. 3. A model for transcriptional regulation by TR. *TR is presumed to form a heterodimer with RXR. The heterodimer binds to the TRE in a target gene. In the absence of T_3, the heterodimer represses gene transcription possibly through the recruitment of a corepressor (C.R.) (e.g., N-CoR, Horlein et al., 1995, or SMRT, Chen and Evans, 1995). The corepressors in turn may facilitate repression by possibly interacting with the transcriptional machinery or forming a repressor complex containing a histone deacetylase (Nagy et al., 1997), which can deacetylate histones as indicated, thus affecting transcription. Upon binding by T_3, a conformational change takes place in the heterodimer, which may be responsible for the release of the corepressor and possibly binding of a coactivator (C.A.) (e.g., SRC, Onate et al., 1995, or CBP/p300, Kamei et al., 1996; Chakravarti et al., 1996), and consequently transcriptional activation as well. Transcriptional activation is also associated with chromatin disruption (Wong et al., 1995, 1997), which may be due to the recruitment of chromatin remodeling factors by TR/RXR or due to the action of the histone acetylase activity of some of the coactivators (e.g., CBP/p300, Ogryzko et al., 1996). This chromatin disruption may be necessary for transcriptional activation by TR/RXR. In addition to TBP, the TATA box binding protein, and RNA polymerase, some other basal transcription factors are also depicted in the figure.*

al., 1994; Baniahmad *et al.*, 1995; Burris *et al.*, 1995; Chen and Evans, 1995; Horlein *et al.*, 1995; Le Douarin *et al.*, 1995; Lee *et al.*, 1995a,b; Onate *et al.*, 1995; Chakravarti *et al.*, 1996; Kamei *et al.*, 1996; Zamir *et al.*, 1996).

How the TR-interacting cofactors participate in T_3-dependent transcriptional regulation remains a mystery. Complicating the matter further is the fact that in eukaryotic cells, the DNA is in association with histones and other nuclear proteins and assembled into chromatin. Increasing evidence suggests that chromatin structure plays important roles in regulating gene transcription (Svaren and Horz, 1993; Kornberg and Lorch, 1995; Lewin, 1994; Wolffe, 1995). In particular, transcriptional activation is often accompanied by chromatin reorganization. One of the best studied examples is the nucleosome remodeling following glucocorticoid induction of MMTV promoter (Pina *et al.*, 1990; Archer *et al.*, 1991; Truss *et al.*, 1995). This hormone-dependent chromatin remodeling allows the binding of the transcription factor NFI, which in turn activates the promoter.

Using an in vivo reconstituted and T_3-dependent transcription system in the *Xenopus* oocytes (Wong and Shi, 1995), we have studied the role of chromatin in transcriptional regulation by TR (Wong *et al.*, 1995,1997). In agreement with studies in tissue culture cells, we have found that both TR and RXR are required for efficient regulation of the T_3-dependent *Xenopus TRβA* gene promoter injected into the oocyte and that TR/RXR heterodimer can repress and activate the promoter in chromatin depending upon the absence and presence of T_3, respectively. Interestingly,

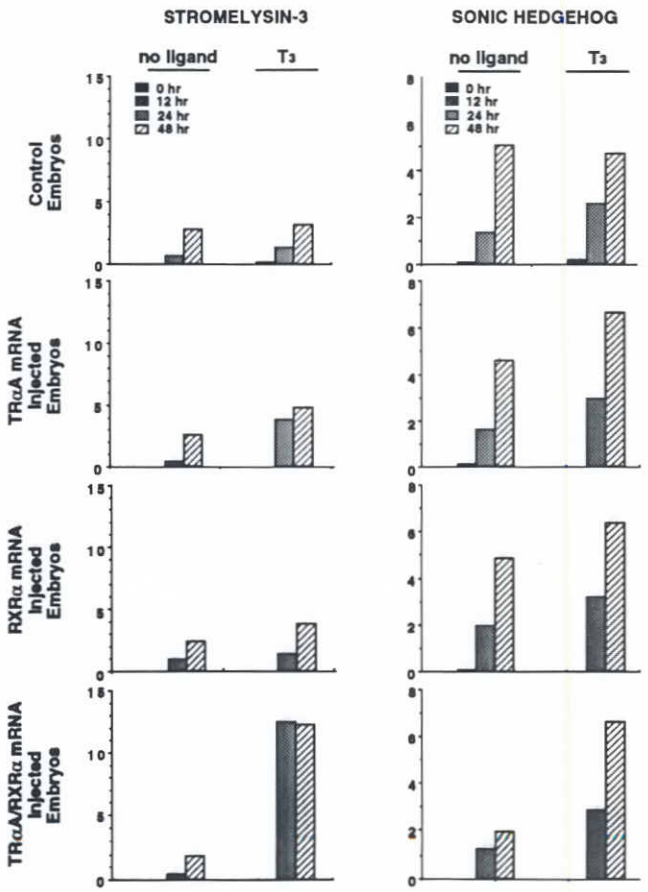

Fig. 4. Over-expression of TR and RXR together but not alone in early Xenopus embryos leads to specific regulation of two T_3-response genes, the *Xenopus sonic hedgehog* and *stromelysin-3* genes (Puzianowska-Kuznicka *et al.*, 1997). *Embryos injected with indicated mRNAs (500 pg per embryo for each mRNA) and cultured in the presence or absence of 100 nM T_3. Total mRNA was isolated and analyzed by Northern blot hybridization. The quantification of the hybridization signals shows that the genes are repressed by the unliganded TR in the presence of RXR and the addition of T_3 leads to the reversal of the repression on both genes and strong activation of the stromelysin-3 gene, in agreement with the observation that stromelysin-3 is up-regulated by T_3 ubiquitously in tadpoles (Wang and Brown, 1993; Patterton et al., 1995) while hedgehog is up-regulated in a few organs (Stolow et al., 1995).*

maximal regulation by T_3 requires the presence of TR/RXR during replication-coupled chromatin assembly *in vivo*. Since in somatic cells TRs/RXRs are present during DNA replication, the results suggest that the oocyte system models nicely the regulation by T3 in somatic cells.

By analyzing the chromatin structure of the *TRβA* promoter injected into the oocyte under various conditions, we have found that while receptor binding in the absence of T_3 has little effect on chromatin structure, the addition of T_3 to chromatin-bound TR/RXR leads to chromatin disruption (Wong *et al.*, 1995,1997). The changes in chromatin are reflected by the increased sensitivity of the minichromosome to micrococcal nuclease and the change in superhelical density of the promoter plasmid purified from the oocyte. Mutational analysis of TR shows that all TR mutants that are capable of activating transcription can disrupt chromatin while those failed to activate the promoter leave the chromatin structure unchanged (Wong *et al.*, 1997), demonstrating a tight correlation between chromatin disruption and transcriptional activation. On the other hand, studies with various mutant promoters in this system show that chromatin disruption alone is not sufficient for transcriptional activation (Wong *et al.*, 1997).

The mechanisms underlying transcriptional activation-associated chromatin disruption are under intense investigation. Studies from yeast to mammals have suggested the involvement of SNF/SWI family of proteins in chromatin remodeling (Yoshinaga *et al.*, 1992; Coté *et al.*, 1994; Imbalzano *et al.*, 1994; Kwon *et al.*, 1994; Tsukiyama *et al.*, 1994,1995; Tsukiyama and Wu, 1995; Varga-Weisz *et al.*, 1995). Similar protein complexes may be involved in chromatin disruption by liganded TR/RXR. In addition, recent evidence suggests that some of the known TR-interacting factors isolated so far may also participate directly or indirectly in chromatin remodeling. For example, the TR coactivator *CBP/p300* has been shown to have histone acetylase activity (Ogryzko *et al.*, 1996). On the other hand, the TR corepressors *N-CoR* and *SMRT* have been shown to interact with the repressor protein Sin3 and form a multisubunit repressor complex that also contains the histone deacetylase 1 (Nagy *et al.*, 1997). These results suggest that histone acetylation and deacetylation are associated with transcriptional activation and repression by TR/RXR, respectively (Fig. 3). This idea is also consistent with the increasing evidence implicating histone acetylation in gene regulation (Wolffe, 1996; Pazin and Kadonaga, 1997; Wade and Wolffe, 1997) and further provide a possible direct linkage of chromatin remodeling and transcriptional regulation. On the other hand, the presence of multiple TR-interacting proteins of yet-unknown functions and our evidence that chromatin disruption is not sufficient for transcriptional activation in at least some circumstances implicate the involvement of multiple pathways in both chromatin remodeling and transcriptional regulation instigated by TR/RXR.

TR/RXR function in developing embryos and tadpoles

Compared to studies in tissue culture systems, relatively little is known about TR function in developing animals. This is in part due to the lack of proper models. The predominant and causative role of T_3 during amphibian metamorphosis makes the tadpole a unique system to investigate how TRs regulate transcription *in vivo*. By microinjecting exogenous genes directly into the caudal skeletal muscle of *Xenopus* tadpoles, De Luze *et al.* (1993) have demonstrated that the endogenous TRs can activate an exogenously

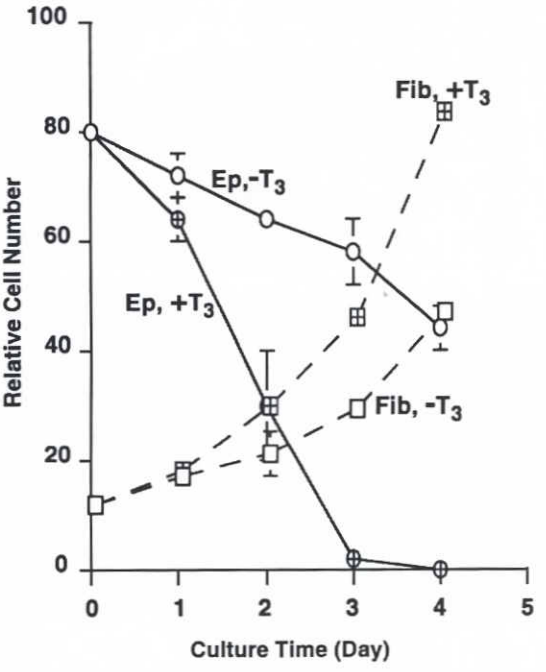

Fig. 5. T_3 induces apoptosis of tadpole intestinal epithelial cells (Ep) but stimulate the proliferation of intestinal fibroblasts (Fib) *in vitro*. *The isolated cells were cultured in vitro in the presence or absence of 100 nM T_3 and live cells were counted after trypan blue staining (Su et al., 1997b).*

introduced gene bearing a TRE, indicating that endogenous TRs in the tadpoles can mediate the effect of T_3 on target gene transcription. Using this method, Ulisse *et al.* (1996) have subsequently introduced dominant-negative mutant TRs together with a reporter under the control of the T_3-dependent *Xenopus TRβA* promoter into *Xenopus* tadpoles. They showed that the dominant negative mutant TRs can block the activation of the *TRβA* promoter, again supporting a functional role of endogenous TRs.

We have made use of the lack of endogenous TR/RXR in early embryos to investigate the function of TR/RXR in development. By microinjecting mRNAs encoding Xenopus TRα and RXRα into fertilized eggs, we have over-expressed TRα and RXRα either individually or together in *Xenopus* embryos (Puzianowska-Kuznicka *et al.*, 1997). The over-expression of individual receptors has little or no effects on embryo development both in the presence or absence of T_3. On the other hand, TRs/RXRs together have severe teratogenic effects on embryonic development if over-expressed at high levels in the absence of T_3. In the presence of T_3, even low levels of TRs/RXRs cause abnormal-development. The phenotypes of the embryos in presence and absence of T_3 are distinct even though some similarities exist, consistent with the fact that TR/RXR heterodimers are transcription repressors in the absence of T_3 and activators when T_3 is present.

More importantly, the expression of several genes known to be regulated by T_3 during metamorphosis is specifically altered by the over-expressed TR/RXR (Puzianowska-Kuznicka *et al.*, 1997). The expression of two such genes is shown in Figure 4. The *stromelysin-3* gene encodes a metalloproteinase that may partici-

pate in extracellular matrix remodeling and is a direct T_3-response gene in all tadpole organs examined (Wang and Brown, 1993; Patterton *et al.*, 1995). The second gene, the *Xenopus hedgehog* gene, encodes a putative morphogen and is a direct T_3-response gene in the intestine but is not regulated by T_3 in most other organs examined (Stolow and Shi, 1995). Both genes are also expressed in early embryos when both TR and T_3 are not yet synthesized (Fig. 4). They are subsequently repressed upon the completion of tadpole organogenesis when tadpole feeding begins at stage 45 and only to be reactivated in all *(stromelysin-3)* or certain *(hedgehog)* organs by T_3 during metamorphosis. The over-expression of TR or RXR alone has little effect on the expression of either gene, independently of T_3 (Fig. 4). However, coexpression of TR and RXR leads to a small but significant repression of the two target genes, especially the *hedgehog* gene, in the absence of T_3 and the addition of T_3 leads to the activation of the *stromelysin-3* gene and only the reversal of the repression of the *hedgehog* gene (Fig. 4). As total embryo RNA was used for Northern blot analysis of the gene expression (Puzianowska-Kuznicka *et al.*, 1997), it may not be surprising to see that the *hedgehog* gene is not up-regulated by the overexpressed TR/RXR in the presence of T_3 since its up regulation by T_3 during metamorphosis is limited to a few organs (Stolow and Shi, 1995). On the other hand, transcriptional repression likely involves different TR/RXR cofactors which may be present in all cell types to mediate the observed repression of the *hedgehog* gene by the over-expressed TR/RXR in the absence of the ligand. These results thus provide strong evidence to support the conclusions that TR/RXR heterodimers are the mediators of the regulatory effects of T_3 and that RXRs are required to efficiently mediate the effects of T_3 during metamorphosis, which was first suggested by the coordinated regulation of TR and RXR genes in different organs during metamorphosis (Wong and Shi, 1995).

In addition, the repression of T_3-response genes by the unliganded, over-expressed TR/RXR suggests that the expression of *TR*α and RXRα in premetamorphic tadpoles prior to the synthesis of endogenous T_3 (Yaoita and Brown, 1990; Wong and Shi, 1995) serves a role to repress the expression of genes that will be needed during metamorphosis. This may be critical to ensure a proper period of tadpole development before changing into frogs since continued expression of these genes may trigger premature metamorphosis.

Conclusions and prospects

We have summarized here some of the evidence implicating a role for *TRs* in amphibian metamorphosis. The correlation of *TR* expression with tissue specific transformation and the functional studies in cell cultures and animals strongly suggest that *TRs* participate in both initiating apoptosis and stimulating the proliferation of adult cell types. The studies on metamorphosis in turn provide one of the strongest in vivo evidence for the requirement of RXR in mediating the effects of T_3 during development, an idea which has been difficult to support with *in vivo* studies (other than in cell culture systems) in mammals.

Both *TR*α and *TR*β are highly expressed during metamorphosis. However, *TR*α mRNAs are present at high levels even in premetamorphic tadpoles (Yaoita and Brown, 1990; Kawahara *et al.*, 1991). This suggests that *TR*α may play a role in premetamorphic tadpoles as unliganded transcriptional repressors to prevent premature expression of genes involved in metamorphosis. *TR*α may also be the primary mediator of T_3 at the onset of metamorphosis when *TR*β levels are low. Both TRα and *TR*β are presumably involved in metamorphosis of different organs once *TR*β genes are activated by T_3. More detailed analyses of temporal regulation of *TR*α and β genes, especially at the protein level, will be needed to determine the roles of different *TRs*.

The functional studies of *TR* action *in vivo* have been limited to a few model systems in amphibians, which include the studies in oocytes (Wong and Shi, 1995; Wong *et al.*, 1995,1997), embryos (Puzianowska-Kuznicka *et al.*, 1997), and tadpole tails (DeLuze *et al.*, 1993; Ulisse *et al.*, 1996). Although oocyte is an atypical cell, the observation that maximal regulation is obtained only when TR/RXR heterodimers are present during replication-coupled chromatin assembly, which mimics the conditions in somatic cells, argues that the conclusion from the studies in oocytes are likely to be true in tadpoles. Similarly, TR/RXR heterodimers over-expressed in embryos can regulate in a T_3-dependent manner the same genes which are regulated by T_3 during metamorphosis when TRs/RXRs are present. This suggests that TR/RXR heterodimers are the mediators of the regulatory effects of T_3 on these genes.

The important future challenge in studying the roles of *TRs* in metamorphosis lies at investigating TR functions directly in metamorphosing tissues/cells. Several potential approaches are now possible. The ability to induce metamorphosis in organ culture with T_3 will continue to facilitate investigations *in vitro*. The recent development of a relative straight forward transgenic methodology in *Xenopus laevis* (Kroll and Amaya, 1996) will greatly improve the possibility to study receptor function in tadpoles. The combination of the transgenic methodology with organ culture technology may further improve the outcome of such studies.

Another approach is to culture primary cell from tadpole tissues and study their responses to T_3 *in vitro*. For example, the tadpole tail epidermal cells (Nishikawa and Yoshizato, 1986; Nishikawa *et al.*, 1989) and intestinal epithelial cells (Su *et al.*, 1997a,b) can be cultured *in vitro* and respond to T_3 similarly as in tadpoles. The intestinal epithelial cells undergo T3-dependent apoptosis *in vitro*. Under the same conditions, the fibroblastic cells from the tadpole intestine are stimulated to proliferate by T_3 (Fig. 5). These differential responses are identical to those observed in the metamorphosing tadpole intestine (McAvoy and Dixon, 1977; Ishisuya-Oka and Shimozawa, 1987), suggesting that these cells will be useful models for studying the signal transduction pathways leading to cell death and proliferation.

Finally, to understand the mechanisms underlying metamorphosis, it is important to study those genes regulated by the receptors. Many such genes have been cloned and encode a variety of proteins including transcription factors, signal transduction molecules, matrix modifying metalloproteinases, and extracellular matrix components, etc. (Shi, 1994,1996; Brown *et al.*, 1996; Gilbert *et al.*, 1996). The critical question is how these diverse groups of T_3-response genes affect downstream events during amphibian metamorphosis, an excellent model system for studying postembryonic vertebrate development.

Summary

Amphibian metamorphosis is an excellent model system for studying postembryonic development in vertebrates. It involves

specific degeneration of larval cells through programmed cell death with apoptotic morphology and selective proliferation and differentiation of adult cell types. Thyroid hormone (T_3) plays a causative role in this process and the effects of T_3 is presumed to be mediated by T_3 receptors (*TRs*). Studies in other systems have suggested that *TRs* function as heterodimers formed with RXRs (9-cis retinoic acid receptors) and require the presence of various cofactor in transcriptional activation and repression in the presence and absence of T_3, respectively. The T_3-induced transcriptional activation leads to chromatin remodeling which may involve some of the cofactors. Recent investigation on receptor expression has implicated a role of *TRs* in T_3-induced apoptosis in larval tissues and proliferation of adult cell types. Functional studies in tadpoles and developing embryos have provide strong support for such a role and further demonstrate the importance of RXR in mediating the effect of T_3.

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