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Review

Molecular and developmental analyses of thyroid hormone receptor function in *Xenopus laevis*, the African clawed frog

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Abstract

The current review focuses on the molecular mechanisms and developmental roles of thyroid hormone receptors (TRs) in gene regulation and metamorphosis in *Xenopus laevis* and discusses implications for TR function in vertebrate development and diversity. Questions addressed are: (1) what are the molecular mechanisms of gene regulation by TR, (2) what are the developmental roles of TR in mediating the thyroid hormone (TH) signal, (3) what are the roles of the different TR isoforms, and (4) how do changes in these molecular and developmental mechanisms affect evolution? Even though detailed knowledge of molecular mechanisms of TR-mediated gene regulation is available from in vitro studies, relatively little is known about how TR functions in development in vivo. Studies on TR function during frog metamorphosis are leading the way toward bridging the gap between in vitro and in vivo studies. In particular, a dual function model for the role of TR in metamorphosis has been proposed and investigated. In this model, TRs repress genes allowing tadpole growth in the absence of TH during premetamorphosis and activate genes important for metamorphosis when TH is present. Despite the lack of metamorphosis in most other vertebrates, TR has important functions in development across vertebrates. The underlying molecular mechanisms of TR in gene regulation are conserved through evolution, so other mechanisms involving TH-target genes and TH tissue-sensitivity and dependence underlie differences in role of TR across vertebrates. Continued analysis of molecular and developmental roles of TR in *X. laevis* will provide the basis for understanding how TR functions in gene regulation in vivo across vertebrates and how TR is involved in the generation of evolutionary diversity.
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1. Hormonal context of TR function in frog development

Metamorphosis in frogs is a post-embryonic developmental process (Tata, 1999) that transforms aquatic, herbivorous tadpoles into terrestrial (usually), carnivorous juveniles (Dodd and Dodd, 1976). Many review articles and books have been written on various aspects of this subject, including hormonal control, molecular and developmental mechanisms, and biochemical and histological metamorphosis of skin, brain, intestine, blood, immune system, liver, and other organs (Allen, 1938; Atkinson, 1994; Balcells, 1955; Brown et al., 1996; Dent, 1988; Denver et al., 2002; Dodd and Dodd, 1976; Etkin, 1964; Galton, 1983; Gilbert and Frieden, 1981; Gilbert et al., 1996; Hourdry, 1993; Kikuyama et al., 1993; Kollros, 1961; Rose, 2005; Sachs et al., 2000; Shi, 1999; Tata, 1996; Wakahara and Yamaguchi, 2001). The intent of this review is to highlight recent molecular studies on the developmental roles of thyroid hormone receptor (TR) in the frog *Xenopus laevis* and how this research informs comparative studies in vertebrate diversity.

Metamorphosis is completely dependent on thyroid hormone (TH) (Gudernatsch, 1912; Allen, 1929). The activities of TH and TR occur in a hormonal context that can be divided into "central" and "peripheral" control of metamorphosis (Fig. 1) (Denver et al., 2002). A key component

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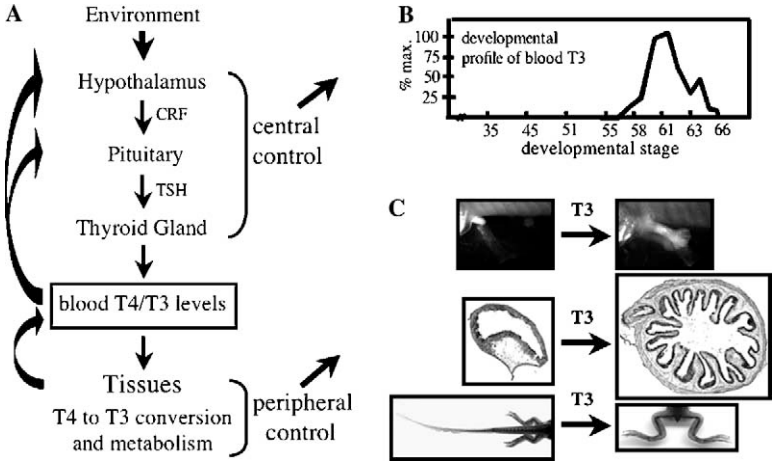

Fig. 1. Endocrine context of TR action and levels of control of metamorphosis. (A) Production of TH by the thyroid gland and tissues responses to TH during metamorphosis constitute the endocrine context of TR. TH is produced by the thyroid gland and secreted into the blood in response to thyroid stimulating hormone (TSH) from the pituitary, which is stimulated by corticotropin releasing factor (CRF) from the hypothalamus. Central control of metamorphosis is the regulation of the levels of circulating TH across development by the hypothalamus, pituitary, and thyroid gland in response to a combination of environmental and nutritional signals and hormonal feedback. Peripheral control of metamorphosis is the tissue-specific responses to TH of tissues outside the hypothalamic–pituitary–thyroid gland axis. (B) The blood levels of TH across development, derived from the thyroid gland and tissue metabolism of T4 to T3, are first detectable in the blood at the point when tadpoles begin to metamorphose at NF stage 55, i.e., when the limbs begin to grow out, and then rise to a peak in the middle of metamorphosis when the morphological transition is most dramatic (Leloup and Buscaglia, 1977). (C) Tissue-specific responses to TH include predominance of cell proliferation in developing limbs (NF stages 51 and 56 are shown). Intestinal remodeling involves both cell proliferation and death in the same tissue (NF stages 54 and 66 are shown in cross-section just posterior to bile duct entry, stained with methyl green pyronine Y). Cell death is predominant during tail resorption (NF stages 57 and 66 are shown).

of metamorphosis, the timing of the peak in TH plasma levels during development, is under central control by the hypothalamus–pituitary–thyroid gland axis (Figs. 1A and B). The neurosecretory hypothalamus, which coordinates environmental and nutritional signals, secretes corticotropic releasing factor into the median eminence, a vascular tissue that supplies the pituitary (Denver, 1999). TH is thought to control the functional development of the median eminence, whose integrity is important for metamorphosis (Etkin, 1965). Corticotropic releasing factor stimulates pituitary thyrotrope secretion of thyroid stimulating hormone (TSH) (Okada et al., 2004), which, in turn, stimulates thyroid follicle cell proliferation and production of TH by the thyroid gland (Kaye, 1961; Sakai et al., 1991). The thyroid gland secretes into the blood mostly thyroxine (T4) and very little of the more active form triiodothyronine (T3) (White and Nicoll, 1981), both forms collectively called TH. TH negatively feeds back on pituitary secretion of TSH (Kaye, 1961; Denver, 1996; Manzon and Denver, 2004). Changes in gene expression of TRs, deiodinases, and receptors for hypothalamic and pituitary hormones likely influence the effectiveness of this feedback (Huang et al., 2001; Manzon and Denver, 2004).

TH target organs outside the central axis, including limbs, intestine, and tail, are collectively known as the "periphery" and respond to T4 and T3 circulating in the blood in a tissue-specific manner (Fig. 1C) (Dodd and Dodd, 1976; Shi, 1999). Some organs develop de novo, such as the limbs, whereas others are completely resorbed, such as the tail and gills (Nakajima et al., 2005). However, most organs, such as intestine and skin, are remodeled from the larval form to the adult version (Fox, 1981; McAvoy and Dixon, 1977; Shi and Ishizuya-Oka, 2001; Suzuki et al., 2002). The only developmental event not known to be affected by TH physiology is primary gonad differentiation (Hoskins and Hoskins, 1919; Gruca and Michalowski, 1961; Ogielska and Kotusz, 2004; Rot-Nikcevic and Wassersug, 2004). Physiological changes accompany the morphological changes, e.g., the transition from ammonotelism to ureotelism, from larval to adult hemoglobins, and from larval to adult immune systems (Gilbert et al., 1996). These tissue-specific developmental events occur asynchronously, e.g., intestine transforms after the limbs and before the tail, and this asynchrony is thought to be due to tissue-specific control of effective intracellular T3 levels (Shi et al., 1996). T4 to T3 conversion by the deiodinase DII in target tissues is one such peripheral mechanism that increases tissue and organ sensitivity to TH (Becker et al., 1997; Cai and Brown, 2004). In addition, T4 and T3 degradation by the deiodinase DIII present in target tissues reduces cellular levels of T3 (Becker et al., 1997). Indeed, transgenic overexpression of DIII blocks TH-induced metamorphic events (Huang et al., 1999; Marsh-Armstrong et al., 1999). Additional cellular proteins controlling intracellular T3 levels and thereby tissue sensitivity to circulating TH, include cytosolic TH binding protein (Shi et al., 1994; Yamauchi and Tata, 1994), TRα (Shi et al., 1996), and TH transporters (Ritchie et al., 2003). TRα expression levels may affect how sensitive cells are to circulating TH. The levels of cytosolic TH binding proteins likely affect free TH within the cells available to bind TR. Furthermore, higher TH transporter expression levels

correlate with increased ability of cells to carry out TR-mediated transcription.

Amphibian metamorphosis offers a unique opportunity to study the molecular mechanisms of TR and interacting cofactors in regulating gene expression in vivo during development. Such in vivo studies have been difficult in mammalian systems because of lack of knowledge about TH-regulated genes during development and the difficulty of obtaining samples from embryos in utero. An additional difficulty in mammalian systems is the inability to study receptor function in plus or minus hormonal states without pathologically disrupting normal development because TH is continuously present, either from the mother or from the fetus. In contrast to mammals, tadpoles are large and accessible throughout their development. In addition, pre-metamorphosis (NF stages 45–53) (Nieuwkoop and Faber, 1994) is characterized by the absence of TH (Etkin, 1932, 1935; Leloup and Buscaglia, 1977), indicating that all of the receptors in vivo are in the unliganded condition. Pro-metamorphosis (NF stages 54–58) and metamorphic climax (NF stages 59–66) have increasing amounts of endogenous TH (Etkin, 1932, 1935; Leloup and Buscaglia, 1977), so that addition of exogenous TH enables precise timing of change to the liganded state that can mimic natural metamorphosis. This ability to control the TH response is a key advantage for using tadpoles, first exploited by showing a role for TH in development and later by isolating TH-responsive genes using subtractive hybridization (Shi, 1999). Recent work has continued to take advantage of frog development as a valuable model of vertebrate developmental endocrinology to address the molecular and developmental roles of TR in post-embryonic development.

2. Molecular mechanisms of TR in gene regulation

2.1. DNA binding, heterodimerization, and role of ligand

Classical in vitro and oocyte studies from a decade ago revealed three critical components for how TR regulates gene expression: DNA binding, heterodimerization, and role of TH (Ranjan et al., 1994; Wong and Shi, 1995; Yen, 2001). TRs are modular proteins consisting of several domains that function largely independently, including DNA and ligand binding domains (Fig. 2A) (Zhang and Lazar, 2000). The 100 amino-acid N-terminal DNA binding domain is responsible for recognizing thyroid hormone response elements (TREs) in promoters or enhancers of TH-regulated genes, while the C-terminal half of the protein binds to the heterodimerization partner (9-*cis*-retinoic acid receptor, RXR), TH, and transcriptional cofactors. TR effects gene transcription by binding to the TREs, the most common of which are direct repeats of AGGTCA separated by four nucleotides, the DR4 type of TREs (Laudet and Gronemeyer, 2002). The TREs in five TH-regulated promoters in *Xenopus* have been characterized to date, TRβ (Machuca et al., 1995; Ranjan et al., 1994), TH-induced basic leucine zipper transcription factor (Furlow and Brown, 1999), basic transcription element binding protein (Furlow and Kanamori, 2002), collagenase or MMP1 (Oofusa and Yoshizato, 1991) and stromelysin-3 (Fu,

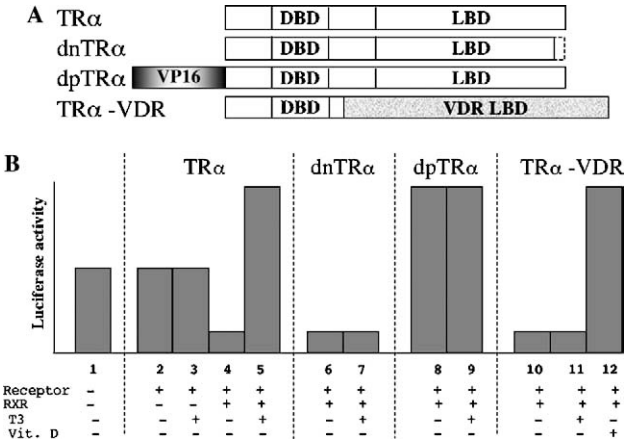

Fig. 2. Role of ligand and receptors in TH-response gene regulation. A good model to study molecular mechanisms of gene regulation is the *Xenopus* oocyte transcription assay because oocytes have large stores of transcription and translation machinery and reporter plasmids become chromatinized when injected into oocytes. This assay involves microinjection of a reporter plasmid (luciferase driven by a TH-inducible promoter as an example here) and transcription factor mRNA into *Xenopus* oocytes followed by luciferase assay to quantitate levels of transcriptional activity. (A) Diagrams of wild type and mutant TRs showing functional domains. Wild type TRα has a DNA binding domain (DBD) and a ligand binding domain (LBD), which is also the site of co-factor binding and heterodimerization with RXR (9-*cis*-retinoic acid receptor). A nuclear localization signal is located in the hinge region between the DBD and LBD. Dominant negative TRα (dnTRα) cannot bind ligand because of a short C-terminal deletion (dashed portion). Dominant positive or constitutively active TRα (dpTRα) has a viral transactivation domain (VP16) fused to the N-terminus that strongly recruits coactivators. The chimeric receptor illustrated here (TRα-VDR) contains the N-terminal portion of TRα, which includes the the DNA binding domain of TRα, fused to the ligand binding domain of the vitamin D receptor (VDR). (B) Because of lack of detectable TR and TH in the oocytes, microinjection of reporter plasmid, containing a TH-responsive promoter controlling the transcription of luciferase, alone into the large oocyte nucleus has a basal level of transcription, as reflected by luciferase activity (lane 1). Basal levels of transcription are not changed upon additional microinjection of TRα mRNA alone into the cytoplasm, with or without T3 (lanes 2 and 3), indicating both the lack of RXR in the oocytes and importance of TRα/RXR heterodimerization in gene regulation by TR. If reporter plasmids and TRα/RXR mRNAs are microinjected into the oocytes, the level of transcription and luciferase activity depends upon the presence of T3, such that in the absence of T3, transcription levels are repressed compared to basal levels, and in the presence of T3, transcription levels are induced above basal levels (lanes 4 and 5). Mutant TRs affect transcription differently with respect to the role of ligand. The dnTRα represses transcription from basal levels, even in the presence of T3 (lanes 6 and 7), because it constitutively binds corepressors due to inability to bind ligand and undergo conformational change. Dominant positive TRα (dpTRα) induces transcription above basal levels even in the absence of hormone (lanes 8 and 9) because of constitutive recruitment of coactivators. This coactivator recruitment even overcomes the possible recruitment of corepressors at the LBD. The TRα-VDR binds to TH-regulated promoters and represses them in the absence and presence of T3 (lanes 10 and 11). Ligand-induced upregulation occurs in the presence of vitamin D rather than T3 (lane 12).

Table 1
TH-responsive promoters in frogs with characterized TREs (all are positive TREs)

Gene	TRE sequence	Position	Cell types	Species
TRβ	AGGTCA TTTC AGGACA[a]	+264 to 280	All cells[f,g]	*Xenopus laevis*
TH/bZIP	GGGTTA AGTA AGGTGA[b]	−99 to 84	All cells[f,h]	*Xenopus laevis*
	AGTTCA AATG AGGCTG[b]	−63 to 78		
BTEB	AGTTCA TCTG AGGACA[c]	∼−6500	All cells[f,i]	*Xenopus laevis*
MMP1	AGGTAA GAAC AGGATA[d]	−891 to 876	Dermis, epi-dermis[j]	*Rana catesbeiana*
ST3	AGGTCA GTTA AGGTGA[e]	+389 to 404[l]	Fibroblasts[f,k]	*Xenopus laevis*

Abbreviations: TH, thyroid hormone; TRE, TH response element; TRβ, TH receptor beta; TH/bZIP, TH-induced basic leucine zipper transcription factor; BTEB, basic transcription element binding protein; MMP1, matrix metalloproteinase 1 (collagenase); ST3, stromelysin-3.

[a] Ranjan et al. (1994).
[b] Furlow and Brown (1999).
[c] Furlow and Kanamori (2002).
[d] Oofusa and Yoshizato (1996) and Sawada et al. (2001).
[e] Fu, unpubl.
[f] Berry et al. (1998a) and Berry et al. (1998b).
[g] Shi and Ishizuya-Oka (1997) and Oofusa et al. (2001).
[h] Ishizuya-Oka et al. (1997).
[i] Hoopfer et al. (2002).
[j] Oofusa and Yoshizato (1991).
[k] Patterton et al. (1995) and Damjanovski et al. (1999).
[l] Within first intron.

unpubl.) (Table 1). In addition, TRs are able to activate transcription through non-DR4 types of TREs (Desvergne, 1994; Williams and Brent, 1995), e.g, in the intestinal alkaline phosphatase gene (Malo et al., 2004) and HIV long terminal repeat (Hsia and Shi, 2002).

The critical roles of heterodimerization of TR with RXR and of ligand can be shown using the frog oocyte transcription assay, where exogenous TR, RXR, and reporter DNA can be introduced into oocytes under conditions mimicking those in somatic cells (Wong et al., 1995). In the absence of T3 and TR, TH-inducible promoters have a basal level of transcriptional activation (Fig. 2B, lane 1). This basal level of transcription is not altered in the presence of TR or T3 (Fig. 2B, lanes 2 and 3), a result consistent with gel mobility shift assays where TRs alone do not bind TREs well in promoters in the absence of heterodimerization (Tsai and O'Malley, 1994; Wong and Shi, 1995). In the presence of TR and RXR, transcription from TH-inducible genes is repressed, and upon addition of T3, transcription is activated (Fig. 2B, lanes 4 and 5).

Mutant TRs with a small truncation or mutations in the C-terminus function as dominant negative TRs because they cannot bind ligand but bind TREs and corepressors normally, thereby repressing TH-inducible genes both in the absence and presence of T3 (Fig. 2B, lanes 6 and 7) (Ulisse et al., 1996). On the other hand, TRs can be converted into dominant positive TRs to activate transcription independent of TH by fusing a strong viral transactivation domain to the N-terminus (Fig. 2B, lanes 8 and 9) (Buchholz et al., 2004). In addition, chimeric receptors, where DNA binding domains and ligand binding domains from different hormone receptors have been swapped, have altered hormone requirements for activation. For example, a chimeric receptor (TRα-VDR) made of the TRα DNA binding domain and the vitamin D receptor ligand binding domain binds TREs but does not respond to TH. However, it activates the TH-regulated genes upon addition of vitamin D (Fig. 2B, lanes 10–12) (Buchholz et al., submitted), indicating that the DNA-binding and hormone-binding domains function largely independently.

Two basic types of transcription response to TH are known, one using positive TREs and the other using negative TREs (Yen, 2001). Positive TREs exhibit the classical case of repression in the absence of TH and activation in its presence, whereas negative TREs behave in the opposite manner. The majority of TH-response genes cloned by subtractive hybridization during frog metamorphosis from intestine (Shi and Brown, 1993), tail (Wang and Brown, 1993), limb (Buckbinder and Brown, 1992), and brain (Denver et al., 1997) are genes upregulated by TH, though a few are downregulated in skin (Furlow et al., 1997) and intestine (Ishizuya-Oka et al., 1994). Many of them appear to be regulated by TH at the transcriptional level and thus likely have positive TREs. On the other hand, TH-response genes analyzed by microarray in mice show a more complicated picture. In liver (Yen et al., 2003) and cerebellum (Miller et al., 2004), most genes are induced by TH. However, in the heart and white adipose tissue, greater than 50% of the genes are downregulated. In the mouse liver microarray study, five modes of TH regulation were observed, classical upregulation and downregulation, derepression only, activation only, and repression only (Yen et al., 2003). Further studies are needed to determine whether these different TH response genes are regulated directly at the transcriptional level through TR or are downstream genes indirectly regulated by T3. Nonetheless, the use of microarray studies in metamorphic tissues are revealing similar complexities in TH-response gene regulation (Veldhoen et al., 2002; Helbing et al., 2003).

2.2. Transcriptional cofactors interacting with TR

The molecular mechanisms of TR in regulating TH-response genes involves cofactors that interact directly or indirectly with TR (Fig. 3A) (Chen and Li, 1998; Ito and Roeder, 2001; McKenna et al., 1999; Xu et al., 1999; Zhang and Lazar, 2000). In the absence of TH, TRs bind corepressors, a number of which have been identified (Burke and Baniahmad, 2000). The best studied among them are the highly related corepressors N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) (Chen and Evans, 1995; Horlein et al., 1995; Privalsky, 2004). When T3 is present, the corepressors are replaced by coactivators including the p160 or steroid receptor coactivator (SRC) family of proteins, p300/CBP (Chakravarti et al., 1996), the DRIP/TRAP/ARC complex (Yuan et al., 1998; Rachez and Freedman, 2001), and chromatin remodeling factors (Huang et al., 2003).

The ligand TH plays an essential role in gene regulation by TR. By means of a CoRNR box (a corepressor/nuclear receptor) consensus sequence, I/L-x-x-I/V-I, (Cohen et al., 2001; Hu and Lazar, 1999), N-CoR or SMRT bind a TR surface formed by helices 1–11 of the C-terminal ligand

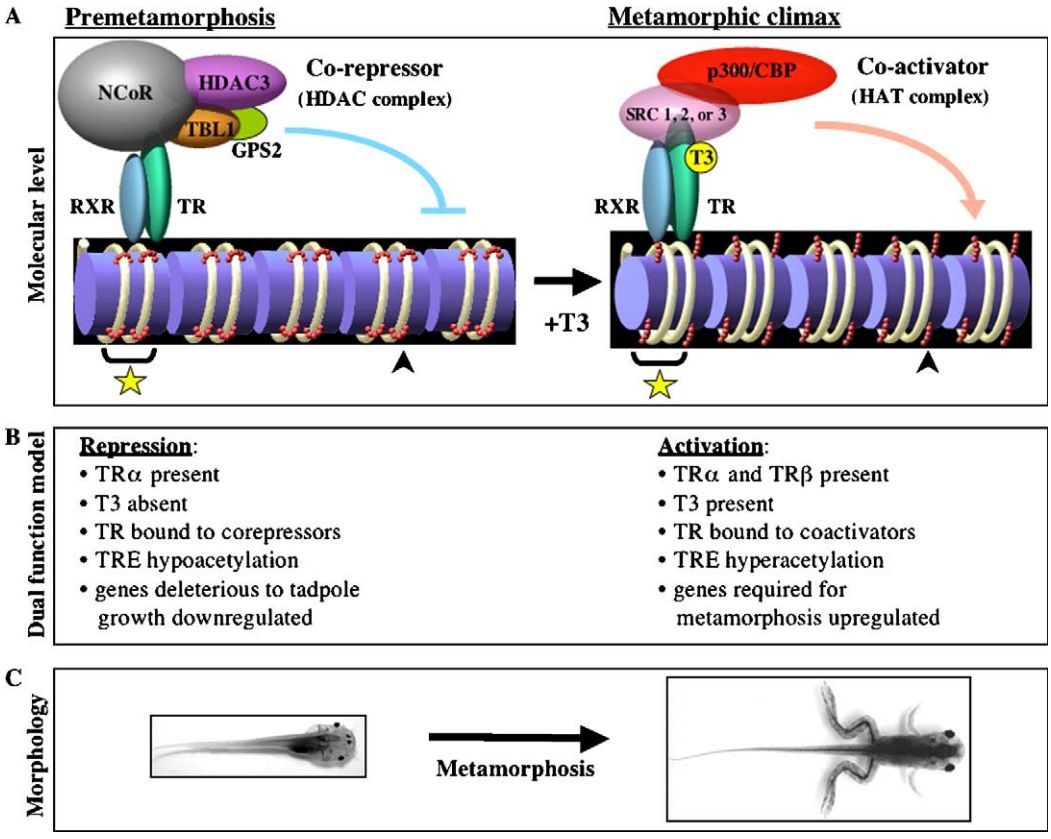

Fig. 3. Dual function model for the role of TR in development and integration of molecular and morphological studies. (A) Molecular studies on premetamorphic and climax tadpoles are consistent with in vitro data showing that TR heterodimerizes with RXR and binds TREs (yellow star) in promoters or enhancers of TH-regulated genes. In the absence of TH, TR binds corepressor complexes, in which corepressors (e.g., nuclear corepressor, NCoR) bind directly to TR and recruit other proteins of the complex to the TRE. Three proteins characterized in the corepressor complex are HDAC3 (histone deacetylase 3), TBL1/TBLR1 (transducin beta-like protein 1 and TBL-related protein) and GPS2 (G-protein pathway suppressor 2). One major activity of this complex is to inhibit transcription from the promoter by histone deacetylation (HDAC) of lysine residues of histone H3 and H4 N-terminal tails (arrowhead), which puts chromatin in a "closed" state for transcription. The presence of T3 induces a change in the conformation of TR conducive to coactivator binding and not corepressor binding. Ligand-bound TR interacts with coactivators, such as steroid receptor coactivator (SRC) 1, 2, or 3. SRCs in turn bind p300/CBP, which has histone acetyltransferase (HAT) activtity to acetylate histones H3 and H4 (arrowhead), which is associated with gene transcription. There are other corepressors and coactivators that bind TR and for simplicity are not shown here. (B) Changes from transcriptional repression to activation precipitates the change from premetamorphosis to metamorphic climax. Numerous studies showed that premetamorphosis is characterized by TRα expression, lack of T3, and little or no expression of TH-inducible genes. In vivo molecular analysis of promoter regions of these genes during premetamorphosis using chromatin immunoprecipitation showed TRα binding to thyroid hormone response elements (TREs), corepressor recruitment, and low levels of histone acetylation, suggesting that TR is acting as a repressor to keep genes important for metamorphosis from being expressed and to allow tadpoles to grow. On the other hand, metamorphic climax is characterized by TRα and TRβ expression, a peak of circulating TH in the blood, and expression of TH-inducible genes. In vivo chromatin immunoprecipitation studies showed TRα and TRβ binding to TREs in promoters, recruitment of coactivators, and high levels of histone acetylation at TH-response genes upregulated during metamorphosis, suggesting that TRs function as activators during metamorphosis at genes important for metamorphosis to proceed. (C) Thyroid hormone controls the transition from tadpole through metamorphic climax to the juvenile. One motivation for developing the dual function model was to bridge the gap in our understanding of how molecular mechanisms of TR bring about metamorphosis at the morphological level.

binding domain (Marimuthu et al., 2002). Within the last 20 C-terminal amino acids of TR is a six amino acid helix (helix 12) not involved in corepressor binding, which, in the presence of T3, folds in and is required for binding T3. In so doing, the corepressor binding surface is obscured (Wagner et al., 1995), and another surface capable of binding the L-x-x-L-L motif of coactivators is formed. Thus, the presence of ligand induces a conformational change in TR causing a switch from corepressor to coactivator binding underlying changes in gene regulation due to ligand (Wagner et al., 1995; Zhang and Lazar, 2000).

Both N-CoR and SMRT exist in multiple histone deacetylase (HDAC)-containing complexes (Jones and Shi, 2003; Li et al., 2000; Yoon et al., 2003), possibly reflecting the fact that numerous transcription factors in addition to TRs utilize N-CoR and SMRT to repress target genes (Glass and Rosenfeld, 2000). Studies in tissue culture cells suggest that the likely corepressor complexes involved in gene repression by unliganded TRs are N-CoR or SMRT complexes that contain HDAC3 (histone deacetylase 3) and TBL1 (transducin beta-like protein 1) or TBLR1 (TBL1-related protein) (Guenther et al., 2000; Li et al., 2000). Subsequently, GPS2 (G-protein pathway suppressor 2) was also shown to be a component of the TR-binding corepressor complex (Zhang et al., 2002). In frog oocytes, TBLR1 associates with unliganded TR in a complex with N-CoR or SMRT at a TH-inducible promoter assembled into chromatin (Tomita et al., 2004). Concurrent with this recruitment, histone acetylation levels at the promoter are reduced, in agreement with the presence of HDAC activity in N-CoR/SMRT-TBLR1 complexes (Guenther et al., 2000; Ishizuka and Lazar, 2003; Li et al., 2000; Yoon et al., 2003). Furthermore, this TR-TBLR1 association is likely through N-CoR or SMRT because a dominant negative form of N-CoR, which consisted of only the TBLR1-interacting domain, was able to inhibit the TR-TBLR1 association in oocytes (Tomita et al., 2004). Thus, unliganded TR appears to recruit N-CoR/SMRT-TBLR1 complexes to deacetylate histones at TH-inducible promoters to mediate gene repression (Fig. 3A).

Among the coactivators that interact with TR directly, the SRC family, which comprise three members, SRC1, SRC2, and SRC3 have been the focus of intense studies (Chen et al., 1997; Hong et al., 1996; Li et al., 1997; Onate et al., 1995; Takeshita et al., 1997; Voegel et al., 1996). The SRC proteins bind TR and other nuclear receptors in a ligand-dependent manner. In frogs SRC3, but not SRC2 and p300, is upregulated by T3 (Paul and Shi, 2003), and SRC binding to TR in the presence of T3 is important for metamorphosis (Paul et al., 2005b). In binding TR, SRCs function as bridging factors to recruit additional cofactors to TH-inducible genes, such as p300, through their p300 interaction domain (Fig. 3A) (Huang et al., 2003). Histone acetylation, carried out by p300, as well as changes in histone methylation and phosphorylation, are associated with transcriptional upregulation by TR (Li et al., 2002). In turn, p300 binds SWI/SNF, which are ATP-dependent chromatin remodeling enzymes (Lemon and Freedman, 1999), and TRIP/DRAP complexes, which interact with and stabilize RNA polymerase II and other basal transcription factors (Ito and Roeder, 2001).

3. Role of TR in frog development

3.1. Dual function model

Our paradigm for understanding the role of TR in frog metamorphosis is encapsulated in the dual function model (Fig. 3B) (Sachs et al., 2000). In vertebrates, there are two loci encoding TR genes, TRα and TRβ, and in mice each gene has multiple splicing variants (Flamant and Samarut, 2003). In psuedotetraploid *Xenopus*, there are duplicates of these, TRαA, TRαB, TRβA, and TRβB (Yaoita et al., 1990). Multiple splicing variants involving the N-terminus exist for TRβA and B but have no known functional difference (Shi et al., 1992). No splicing variants are known for TRα isoforms in *Xenopus*. During development, TRα and RXRs are expressed throughout the larval period from the beginning of feeding onward well before the production of TH, whereas the presence of TRβ is correlated with TH levels (Baker and Tata, 1990; Helbing et al., 1992; Kawahara et al., 1991; Luria and Furlow, 2004; Yaoita et al., 1990). However, the existence of a specific embryonic expression site for TRβ has been identified in the developing retina (Cossette and Drysdale, 2004), but this TR may utilize TH deposited in the yolk and not be involved in metamorphosis. Explanation for the TH-independent timing of TRα expression prompted formation of the dual function model to bridge the gap between molecular mechanism and morphological outcomes (Fig. 3C).

The dual function model incorporates results from in vitro studies, indicated above, showing that TR functions as a repressor of TH-regulated genes in the absence of hormone, and in the presence of hormone, TR functions as an activator of these same genes. This dual effect of TR on gene regulation in vitro was applied to development, with its expression pattern of TRs and its profile of circulating TH levels, to generate the hypothesis that TRs mediate repression during premetamorphosis in the absence of TH and activation during metamorphosis when TH is present. For both repression and activation, the dual function model is a hypothesis of (1) the molecular activities of TR in vivo in the presence and absence of T3, as well as (2) the consequences of this gene regulation for development. Another sense of a dual function, not implied here, refers to two potential effects of early TRα expression before metamorphosis, where TRα might repress metamorphic genes until needed and make cells sensitive to T3 so they can respond to the hormone in the first place.

At the molecular level of gene regulation, the dual function model states that when TH is absent during premetamorphosis, TR functions as a repressor, predominantly TRα. Then, upon secretion of TH from the thyroid gland, TRα binds the hormone and recruits SRC3 and other coac-

tivators, thereby becoming a transcriptional activator that induces target gene expression. The activation of TRα induces high expression of TRβ, which itself interacts with coactivators due to the presence of TH. Thus, the predominant role of TRβ in gene regulation is in activation, not because it cannot interact with corepressors, but because TRβ is expressed for the most part only when T3 is present. The switch from corepressor to coactivator binding results in changes in histone acetylation and chromatin remodeling favoring gene activation. The molecular aspects of this model apply to the mechanisms of TR in regulating direct response genes, i.e., genes where TR binds TREs in the promoter, as opposed to downstream or late genes in the TH-induced gene regulation cascade (Shi, 1994).

The dual function model at the developmental level suggests that TR-mediated repression is important to keep metamorphic genes turned off to allow tadpole growth and prevent metamorphosis from occurring too early. This includes several genes expressed during embryogenesis that are also involved in metamorphosis, such as stromelysin-3 and sonic hedgehog (Ishizuya-Oka et al., 2000, 2001; Stolow and Shi, 1995). Thus, early TR expression may be important to turn off these embryonic genes until they are again required for metamorphosis. Furthermore, gene activation in the presence of T3 is important to initiate the morphological and physiological changes of metamorphosis. The developmental aspects of the model apply to the consequences for development of TR-regulated genes expression.

Two considerations are important when discussing the applicability of the dual function model. First, consequences of the conformational change induced by TH causing TR to switch from a repressor to an activator may depend on the promoter. Given the variety of gene regulation patterns directly mediated by TR including TH-induced gene activation or repression (Yen et al., 2003), the dual function model may not represent all activities of gene regulation mediated by TR. The dual function model was originally conceived to apply to positive TREs. For the genes that are only activated or only repressed by TR, other models of gene regulation are needed to explain how a TH-response gene can be, for example, activated and not repressed by TR. For direct response genes that are downregulated, such as TSH in the pituitary (Manzon and Denver, 2004), the dual function model may apply but just be opposite in sign. However, the molecular mechanisms are not understood in vitro for TR-mediated regulation of genes other than those with positive TREs, so a model for the developmental role of TR for regulating genes with other types of TREs must await biochemical characterization.

Second, the dual function model may also have to be understood in the context of tissue-specific gene regulation. For some genes that are up regulated in all cells, such as TRβ, TH-induced basic leucine zipper transcription factor, and basic transcription element binding protein (Table 1), we suggest the dual function model applies similarly to these genes in all cells. Other genes are upregulated in a broad organ distribution but in particular cell types, such as stromelysin-3 in fibroblasts (Table 1), or expressed in a specific tissue and organ, such as sonic hedgehog in intestinal epithelium (Patterton et al., 1995; Stolow and Shi, 1995). For these genes, the dual function model applies only in the cell-type in which they are expressed. In addition to tissue-specificity are temporal considerations. Metamorphic events occur asynchronously, indicating that the transition from repression to activation of the dual function model occurs at different times in different tissues during development. For example, the hind limbs develop before the tail resorbs, suggesting that TR is an activator in the hind limbs at the same time it is a repressor in the tail, which may have to do with intracellular levels of ligand owing to deiodinase expression.

3.2. In vivo support for molecular aspects of the dual function model

Support has come from analyses of the expression of TR and cofactors by us and others. We have found that corepressor components N-CoR, SMRT, and TBLR1 (Sachs et al., 2002; Tomita et al., 2004) and GPS2 and HDAC3 (Buchholz et al., submitted) are expressed during premetamorphosis, when they may be expected to participate in gene repression by the unliganded TRα and RXR present at stages prior to T3 production (Yaoita and Brown, 1990; Wong and Shi, 1995). Using the chromatin immunoprecipitation assay (Fig. 4A) (Damjanovski et al., 2002; Das et al., 2004; Spencer et al., 2003), we have shown that TR, as well as the corepressors N-CoR, SMRT, and TBLR1, are bound to TH-responsive promoters in vivo in premetamorphic tadpoles (Buchholz et al., 2003, 2004; Havis et al., 2003; Sachs et al., 2002; Sachs and Shi, 2000; Tomita et al., 2004). Similarly, coactivator components such as SRC 2 and 3 and CBP/p300 are expressed throughout the larval period, and importantly for the dual function model, during metamorphosis (Paul and Shi, 2003). In addition, the chromatin immunoprecipitation assay shows they bind to TREs in vivo during metamorphosis or when premetamorphic tadpoles are treated with TH (Havis et al., 2003; Paul et al., 2005a; Paul et al., 2005b). Furthermore, histone acetylation levels are lower in the absence of TH than in the presence of TH at TH-response genes in vivo, in agreement with the proposed role of histone deacetylases and histone acetyltransferases in gene repression and activation by TR, respectively (Sachs and Shi, 2000).

3.3. Evidence for the existence of TR repression in vivo

As mentioned above, some TH-response genes, such as stromelysin 3 and sonic hedgehog, are expressed during embryogenesis when there is little TR and RXR and also during metamorphosis (Patterton et al., 1995; Stolow and Shi, 1995). During the intervening premetamorphic period,

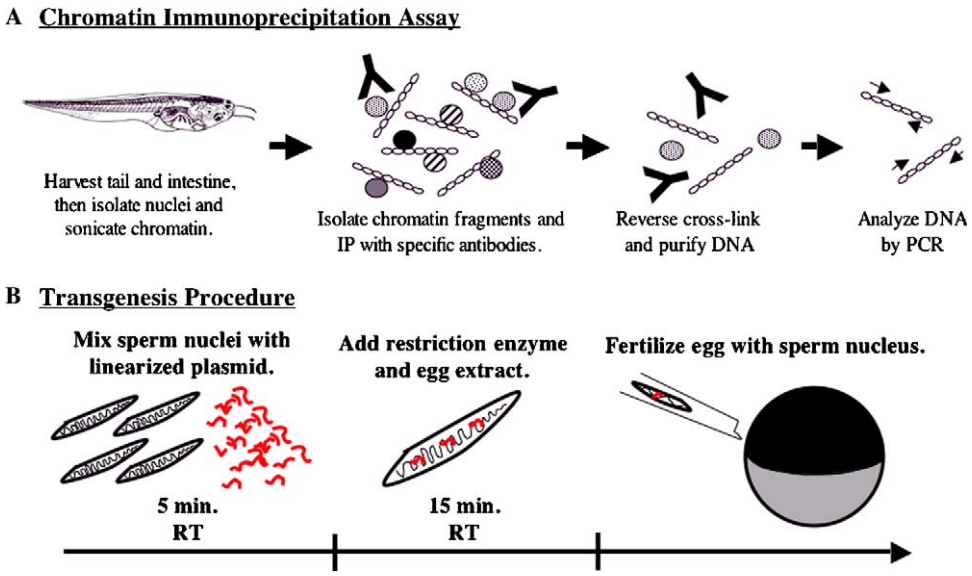

Fig. 4. Techniques important for in vivo molecular analysis of the role of TR during development. (A) The chromatin immunoprecitation assay directly measures protein–DNA interactions in cells and takes advantage of the specificity of antibodies and the sensitivity of PCR. For in vivo analysis, tadpole tissues are harvested and the nuclei are isolated. The nuclei are then lysed with detergent, and the intact genomic DNA is sonicated to produce small pieces of chromatin (~200–1000 bp in length). Antibodies specific for a protein of interest, e.g., anti-TR antibodies or antibodies against a cofactor that interacts with TR, are added to the chromatin to immunoprecipitate (IP) fragments of DNA that may be associating with the protein of interest. Then, the immunoprecipitated DNA is purified and PCR is carried out with primers specific for a TH-responsive promoter to detect whether or not the protein of interest was associated with that promoter region. The chromatin immunoprecipitation assay always involves two chromatin samples from, for example, premetamorphic tadpoles treated with or without T3. In this example, anti-coactivator antibodies would not yield a PCR product in the untreated tadpoles because in the absence of T3, TR does not bind coactivators. However, in the presence of T3, corepressors are dissociated from the TR and replaced by coactivators so that the chromatin from the T3-treated tadpoles would give a strong PCR product with the anti-coactivator antibodies. (B) The restriction enzyme-mediated integration method produces transgenic frogs with transgenic DNA inserted randomly, yet stably, into the genome throughout development from the first cell stage on. Sperm nuclei prepared from the testis are mixed with linearized plasmid containing a promoter controlling expression of the transgene. Then restriction enzyme and egg extract are added to swell the nuclei and aid in plasmid integration. These treated nuclei are then injected into freshly ovulated eggs to fertilize them. Surviving transgenic frogs can be detected by PCR typing or by use of a cassette of crystallin promoter driving green fluorescent protein in the eyes included in the transgenesis plasmid that results in transgenic tadpoles with green eyes (Fu et al., 2002).

i.e., after tadpole feeding begins until the onset of metamorphosis, these genes are expressed at lower levels. This interval between the expression peaks of these genes corresponds with the expression of TRα and the absence of T3, suggesting that TRα may repress these genes. Similarly, the downregulation of the transgene GFP under control of the TRβA promoter correlated with the expression of TRα in the absence of T3 (Oofusa et al., 2001). Embryo injection experiments have provided further evidence to show that TR is capable of repressing or activating gene expression in developing *Xenopus* embryos (Puzianowska-Kuznicka et al., 1997). Overexpression of TR and RXR together, but not either one alone, by microinjecting their mRNAs into fertilized eggs was shown to repress endogenous TH response genes while the addition of TH led to the reversal of the repression and further activation of these genes, suggesting that subsequent expression of endogenous TR might repress these genes. Similarly, endogenous TR and cofactors are capable of repressing reporter genes, as suggested when dominant negative N-CoR peptides caused upregulation of a co-injected reporter gene in tadpole tail cells in vivo (Sachs et al., 2002). According to the developmental aspects of the dual function model, we expect TR/corepressor complexes to repress genes that, if expressed, would cause metamorphic defects. In support of this, functional studies show deleterious consequences of precocious expression of the TH-regulated gene stromelysin 3 in transgenic animals where normal intestine morphology is compromised in the absence of T3 (Ishizuya-Oka et al., 2000; Fu et al., 2005).

Correlations between TR expression and TH-response gene expression, as well as embryo and tail injections studies, need to be augmented by direct experimental evidence in order to establish endogenous TR is responsible for repression of endogenous genes, for example, by blocking corepressor function at the TR with overexpressed transgenic dominant negative corepressors. Two pieces of indirect evidence suggest that TR-mediated repression may play only a minor role in *Xenopus* post-embryonic development. First, we used a dominant negative form of *Xenopus* SRC3 (F-dnSRC3) overexpressed in transgenic animals to investigate the function of coactivators during metamorphosis, and we showed that this overexpression was sufficient to inhibit or delay both TH-induced and natural metamorphosis (Paul et al., 2005b). Despite the corepressor release in these animals treated with T3, little derepression

and tissue transformation were observed, suggesting that TR/corepressor complexes were not the major factors responsible for repressing these genes in premetamorphosis. Second, in vivo TR binding to the TH/bZIP promoter is low or not detectable in the absence of T3 but increases dramatically in the presence of hormone, whereas high TR binding to the TRβ promoter was constitutive (Buchholz et al., submitted). These results suggest that at least for some genes, sufficient amounts of TR may not be present at the promoters to mediate gene repression so that unliganded TR binding and repression during premetamorphosis is likely gene-specific.

3.4. Evidence for a developmental role of activation in vivo

The transformations induced by TH during metamorphosis are believed to be mostly, if not completely, mediated by TR, although non-genomic effects of TH are known to exist (Davis and Davis, 1996). Several types of experiments strongly support the model that TR-mediated activation is necessary and sufficient to initiate metamorphic events. Indirect evidence for the role of TR in the TH response comes from the correlation of TR expression with metamorphosis, where TR expression is highest at climax when TH levels are highest (Yaoita and Brown, 1990). Also, exogenous TH added before TR expression does not induce metamorphic transformation suggesting the importance of TR (Tata, 1968). In addition, chromatin immunoprecipitation experiments show TR binding directly to promoters of TH-regulated genes during metamorphosis (Buchholz et al., 2003; Sachs and Shi, 2000). Furthermore, overexpression of TR and RXR in developing embryos through microinjection of mRNAs into fertilized eggs disrupts development in a TH-dependent manner (Puzianowska-Kuznicka et al., 1997), indicating that inappropriate regulation of TH-response genes has deleterious effects.

Direct evidence for a role of TR in metamorphosis comes from transgenic tadpoles overexpressing mutant TRs, either dominant negative (dn) or dominant positive (dp) forms of TR. Overexpression of these mutants in tadpoles is accomplished by restriction enzyme mediated integration method of frog transgenesis (Fig. 4B) (Fu et al., 2002; Kroll and Amaya, 1996). In transgenic tadpoles overexpressing a dnTR under control of the CMV promoter which promotes high expression levels in all cell types, action of T3 is blocked (Fig. 5) (Buchholz et al., 2003; Schreiber et al., 2001). This dnTR constitutively recruits corepressors to TREs thereby inhibiting both activation, as well as derepression, and thus these experiments show that gene activation and/or derepression is required for metamorphosis. Experiments using dnSRC3, as discussed above, resolved this issue, by showing that activation, in addition to derepression, is required for metamorphosis (Paul et al., 2005b). These studies thus demonstrate the requirement for TR to mediate the T3 signal during metamorphosis.

Studies with a dpTR show that the receptor is sufficient to mediate the effect of TH to initiate metamorphosis, i.e., TH does not seem to signal through other pathways (Buch-

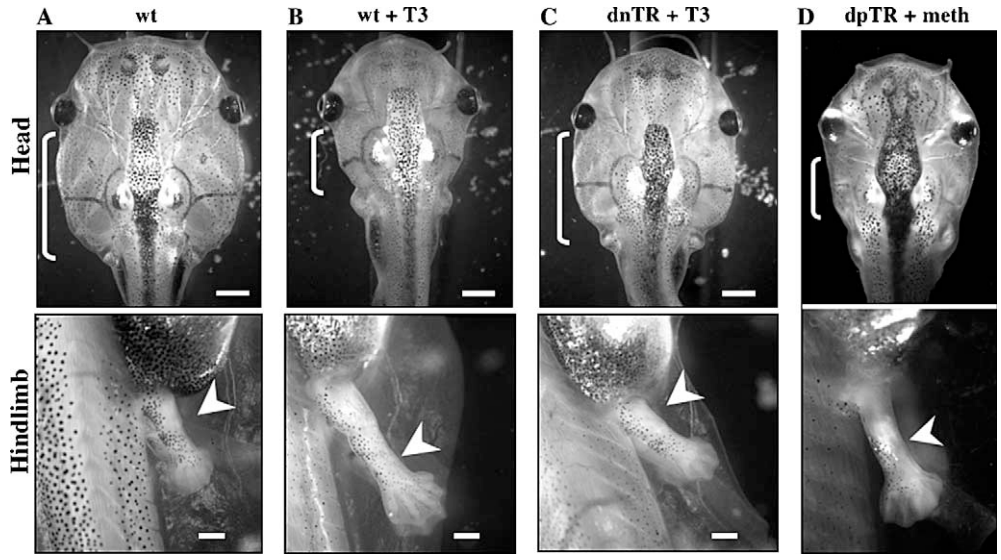

Fig. 5. Transcriptional activation through TR is necessary and sufficient to mediate the TH signal initiating metamorphic events. (A) During premetamorphosis, tadpoles have well-developed gills (bracket) and short limbs with undifferentiated digits (arrowhead). (B) After 3 days of exogenous T3 treatment (5 nM at 18 °C), precocious metamorphosis occurs as seen by gill resorption (bracket) and limb outgrowth (arrowhead). (C) In transgenic tadpoles, overexpressed dominant negative TR (dnTR) blocks TH-response gene activation by competing with endogenous TRs for binding to TREs, and blocks to a great extent metamorphic changes in gill resorption (bracket) and limb growth and differentiation (arrowhead). This experiment shows that activation function of TR is necessary for metamorphic progression. (D) Transgenic tadpoles with a dominant positive TR (dpTR) under control of a heat-shock inducible promoter are reared in methimazole (meth), a chemical that blocks endogenous TH synthesis. Heat shock induction of dpTR expression results in metamorphic events, including gill resorption (bracket) and limb outgrowth (arrowhead). Because dpTR expression induces metamorphic events in the absence of T3, this experiment indicates that transcriptional activation through TR is sufficient to initiate metamorphosis, i.e., the TH signal goes through TR rather than some other signalling pathway for the metamorphic events measured.

holz et al., 2004). Because early embryogenesis may be affected by inappropriate TR function (Puzianowska-Kuznicka et al., 1997; Wong and Shi, 1995), dpTR was put under control of a heat shock-inducible promoter to control the timing of transgene expression (Fu et al., 2002; Wheeler et al., 2000). In addition, to rule out potential effects from endogenous TH, transgenic tadpoles were reared in methimazole, a chemical that blocks endogenous TH synthesis (Buckbinder and Brown, 1993). Upon heat shock to induce transgene expression, dpTR transgenic tadpoles at premetamorphic stages underwent metamorphosis in the absence of hormone like the wild type siblings treated with T3 (Fig. 5). It is important to note that dpTR expression and exogenous T3 share similar phenotypes, in that, unlike natural metamorphosis, induced metamorphosis is characterized by simultaneous transformation of TH-responsive tissues and a lack of normal developmental asynchrony. Furthermore, dpTR was shown to bind endogenous TH target promoters, and all TH-response genes, both early and late, were found to be regulated as in tadpoles treated with TH. These studies indicate that TR is sufficient to mediate the effects of exogenous T3 on all metamorphic parameters that were measured.

3.5. Molecular and developmental roles of TR isoforms

The *Xenopus* TRα and TRβ have no known functional differences in transcription regulation in vitro or in vivo at the molecular level (Puzianowska-Kuznicka et al., 1997; Wong and Shi, 1995). Oocyte assays using either TRα or TRβ show no difference in transcription from the TRβ promoter (Wong and Shi, 1995). In vivo, both TRα and TRβ bind T3-regulated promoters, and TRα can activate all tested direct response genes, based on TRα transgenic receptors (Buchholz et al., 2004). In addition, Scatchard analysis indicates no difference in affinity between TRα and TRβ for binding to TREs of TRβ and TH/bZIP promoters, though both TRs bind the TRβ promoter with 4-fold higher affinity than the TH/bZIP promoter (Buchholz et al., submitted). However, experiments from mammalian TRα and TRβ domain swapping studies suggest differences in gene regulation from a negative TRE, though no differences were found on positive TREs (Guissouma et al., 2002). Thus, differences in molecular mechanisms of TRα and TRβ in gene regulation at different promoters through the divergent N-terminal domains of the TRs cannot be ruled out completely, although the observed differences between the TRs may be due to in vivo post-translational modification differences between TRα and TRβ leading to different levels of the receptors under the assay conditions of the mammalian study (Guissouma et al., 2002). Even though, for the most part, TRα and TRβ seem to have similar molecular roles at TH-response promoters, they likely have different roles in development because of differences in the TH inducibility of their expression and other unknown aspects of the TR promoters controlling their tissue-specific differential expression.

As detailed above, the temporal expression profiles of TRα and TRβ led to the dual function model. TRα protein levels are 2–3-fold more than TRβ in the head or tail at premetamorphosis and do not change significantly as metamorphosis proceeds, whereas TRβ protein expression dramatically increases through metamorphic climax (Eliceiri and Brown, 1994). This major difference between the TRs may be explained by the presence of a TRE in the TRβ promoter, which is induced by the T3 present during metamorphsis (Machuca et al., 1995; Ranjan et al., 1994). Consequently, at metamorphic climax, TRβ protein levels exceed TRα levels in both head and tail (Eliceiri and Brown, 1994). Thus, TRα may serve predominantly as the repression arm of the dual function model, whereas the feed forward autoregulation of TRβ expression (Tata, 1994; Yaoita and Brown, 1990) may ensure that TRβ plays a critical role in carrying out activation during metamorphosis.

The increase in TRβ levels, or autoregulation, is correlated with metamorphosis (Shi and Ishizuya-Oka, 1997), though direct experimental support for the importance of autoregulation is lacking. Indirect evidence comes from the near background levels of TR binding to the TH/bZIP promoter in premetamorphosis due to a lower affinity TRE compared to the TRE in the TRβ promoter (Buchholz et al., submitted). Only an increase in TR expression levels by autoregulation will allow lower affinity TREs to be occupied enabling their upregulation during metamorphosis.

Another difference between receptors is tissue differences, i.e., different tissues may have different relative amounts of TRα vs. TRβ not explained by the TRβ promoter TRE. For example, in the hind limb, high TRβ mRNA expression is localized to a subset of cells during transformation, whereas TRα mRNA is spread throughout (Fairclough and Tata, 1997; Rabelo et al., 1994). Also, in the tadpole brain, proliferating cells in the subventricular zones express high levels of TRα mRNA, whereas these cells do not express TRβ (R.J. Denver, pers. comm.) Cells distal to the ventricles that are destined to differentiate or undergo apoptosis express TRβ. Use of an isoform-specific agonist, GC-1, showed developmental disregulation different from that observed upon T3 treatment (Furlow et al., 2004). In vitro, GC-1 preferentially activates TRβ over TRα with a 10-fold difference in selectivity. Treatment of tadpoles with GC-1 leads to tail and gill resorption with lesser effects on hind limb outgrowth. These organ-specific responses can be explained by tissue-specific TRα expression levels in premetamorphosis and/or tissue-specific ability for TRβ autoinduction. The tail resorbs and limbs do not in GC-1-treated tadpoles either because the high TRα in the limbs (Kawahara et al., 1991; Cai and Brown, 2004) acts as a dominant negative against the small amount of TRβ that is induced by GC-1in the limb or because the limbs do not strongly induce TRβ. In the tail, the small amount of TRβ upregulated by GC-1 can continue autoregulation without much competitive inhibition from low endogenous

levels of TRα and therefore carry out the GC-1-induced transformation. These examples suggest the two TR isoforms have different developmental roles, in that TRα is associated with proliferating cells and TRβ with cell differentiation, though high levels of TRβ are correlated with both larval epithelial cell death and adult epithelial cell proliferation in the intestine (Shi and Ishizuya-Oka, 1997). Thus, the similarities in molecular action of TRα and TRβ at the level of gene regulation contrast with the divergent roles of the TR isoforms in development due to differential control of the expression levels in and across tissues. On the other hand, because it seems necessary to have proliferative events precede resorptive or differentiative events in metamorphosis, the TR isoforms, with their differential expression temporally and spatially due to tissue-specific levels of TRα expression and TRβ autoinduction, may act, not divergently, but coordinately as another mechanism to control developmental timing of metamorphic events (Shi et al., 1996).

3.6. Tissue-specific consequences of TR activation

Different cell types have very different responses to TH activation, e.g., cell death in the tail and cell proliferation in limbs and both death and proliferation in remodeled organs (Dodd and Dodd, 1976). These different cell fates suggest tissue-specific gene regulation cascades induced by TR. Many TH-regulated genes have been isolated by subtractive hybridization from intestine (Shi and Brown, 1993), tail (Wang and Brown, 1993), limb (Buckbinder and Brown, 1992), and brain (Denver et al., 1997), and many more are now being identified by microarray technology (Helbing et al., 2003; Veldhoen et al., 2002). Even though many of these genes are similarly upregulated in all tissues, e.g., TRβ and TH-responsive basic leucine zipper transcription factor, unique cell expression patterns among other TH-response genes, such as sonic hedgehog in the intestinal epithelium, may underlie the differing cell fates among tissues. Indeed, in situ hybridization analysis of TH-induced genes in the tail (Berry et al., 1998b) and head (Berry et al., 1998a), reveal cell type-specific expression patterns of many induced genes within these body regions.

The extent of cell autonomy of the tissue-specific responses to T3 has been probed using transgenic approaches to block the action of T3 in a particular cell type by overexpressing dominant negative TRs or DIII with tissue-specific promoters. Both cell autonomous events and events requiring TH-activation of other cell types have been identified (Nakajima et al., 2005). Overexpression of a dominant negative TR under control of a larval keratin promoter directing expression specifically in larval epidermis, inhibited the death of these cells in response to T3 without affecting adjacent fibroblasts or formation of adult skin (Schreiber and Brown, 2003). Using a muscle-specific promoter expressing dominant negative TR, early TH-induced death in muscle cells in the tail was delayed until the tail shortened later in metamorphosis by inducing non-cell autonomous muscle cell death (Das et al., 2002; Yaoita and Nakajima, 2003). Also, muscle cells of the limbs were very poorly developed, even though bones, nerves, and skin of the limb was normal. These experiments revealed the cell autonomous nature of early tail muscle death and limb muscle growth, which is independent of TH action on other cell types. In addition, non-autonomous development was observed in that tail muscles eventually died, presumably by breakdown of connective tissue around the muscle cells caused by TH-activated fibroblasts. Similarly, dominant negative TR overexpressed in neurons showed cell autonomous development of spinal cord cells innervating the limbs (Marsh-Armstrong et al., 2004). Further evidence for the importance of both cell autonomy and cell–cell interaction in TH-induced transformation came from in vitro tissue recombination studies of the intestine where the importance of intercellular signals in both directions between epithelium and connective tissue were observed (Ishizuya-Oka and Shimozawa, 1992, 1994).

4. Implications of TR studies in X. laevis

To what extent can we use our knowledge of TR function during metamorphosis of *X. laevis* to help understand vertebrate development and the evolution of vertebrate diversity? Among vertebrates, metamorphosis, characterized by a peak in TH levels and a sudden larval to juvenile transition, seems to be found in three distantly related groups from each other, namely, amphibians, Pleuronectiformes (flatfish), and Elopimorpha (eels and relatives) (Norris, 1983; Tagawa et al., 1990; Youson, 1988). Lampreys undergo TH-dependent metamorphosis, but the larval to juvenile transition is precipitated by a drop in TH levels, rather than a peak (Youson, 2003). Many groups of teleosts have larvae with an extended transition period where larval traits are gradually transformed to adult versions (Urho, 2002; Youson, 1988), in some ways resembling the development in utero or in ovo of mammals, birds, and reptiles.

Despite lack of a larval period and metamorphosis in most vertebrates, all vertebrates are believed to require TH for normal post-embryonic development. In fact, a peak in plasma TH concentration associated with thyroid hormone-dependent development occurs across vertebrates, such that developmental endocrinology studies in *Xenopus* may contribute knowledge about development of all vertebrates. In mice, the period of weaning characterized by a change in diet from milk to adult food is associated with a peak in thyroid hormone (Hadj-Sahraoui et al., 2000; Henning, 1987), and the accompanying intestinal remodeling at weaning has been shown by hormone manipulation studies and TR gene knock mice to be dependent on TH (Plateroti et al., 1999). A peak in TH also occurs in humans at birth (Fisher, 2002) and in some fish, such as salmon, during smoltification (Norris, 1996), a transi-

tion period in physiology from freshwater parrs to saltwater smolts. However, the role of TH physiology during these periods in humans and fish is not clear. An important feature of detailed knowledge of TR function in *Xenopus* lies in the ability to identify possible changes in molecular mechanisms and developmental roles of TH and TR that may have contributed to the large diversity of morphology and physiology across vertebrates (for extended reviews of these ideas applied to salamander larval diversity, see Rose, 1996, 1999). First, we discuss whether the dual function model applies to vertebrates without metamorphosis, and then we suggest potential changes in the developmental role of TR, including changes in tissue sensitivity, response, and dependence, that may underlie evolutionary diversity.

4.1. Dual function of TR during development in other vertebrates

Comparisons between molecular mechanisms characterized in frogs and mammalian cell culture studies, such as histone acetylation and cofactor recruitment, reveal an emerging picture of similarity across vertebrates at the level of molecular mechanisms controlling gene regulation at the promoter (see detailed examples above). The molecular components that interact with TR in frogs are homologous to those in Hela cells and mice, i.e., similarities are the rule for TRE binding, cofactor recruitment, chromatin remodeling, and role of ligand. Does this similarity in molecular mechanisms of TR in gene regulation extend to a dual function model for development applicable across vertebrates, where TR functions as a repressor to downregulate genes important for development, which are subsequently activated via TR upon the presence of T3? Indirect evidence from mouse knockout studies suggests the dual function model does apply. TR double knockouts have a less severe phenotype than hypothyroid mice, suggesting that uncontrolled, TR-mediated repression has deleterious developmental effects (Flamant and Samarut, 2003). Also, overexpression of a dominant negative N-CoR in liver parenchyma upregulated liver TH response genes, indicating that TR mediates both repression and activation in the liver (Feng et al., 2001; Yen et al., 2003). More importantly, during normal mouse development, unliganded TRα represses heart rate in prenatal embryos, accompanying the repression of TRβ and several genes encoding ion channels involved in cardiac contractile activity, and at or after birth when the T3 levels are high, liganded TRα turns on the expression of some of these same genes concomitantly with heart rate increase (Mai et al., 2004). These results indicate that dual functions of TR are important for normal mouse development as proposed for frog development.

4.2. Tissue sensitivity

Because molecular mechanisms of TR-mediated gene regulation are shared across vertebrates, it is unlikely that explanations for developmental differences between species will be found in molecular differences in TR function. Rather, developmental aspects of TR will contribute to vertebrate diversity. Differential timing of developmental events with respect to each other within *Xenopus* is well known, where the hind limbs develop before intestine, and this is believed be due, at least in part, to differential tissue sensitivity to TH, i.e., tissue-specific control of the switch from TR repression to TR activation as a result of tissue-specific levels of TRα, cytosolic TH binding protein, type II deiodinases, and/or type III deiodinases (Becker et al., 1997; Shi et al., 1996). Differential expression of these proteins that control developmental timing within species may have changed during evolution to underlie differences in timing of developmental events across species, for example, the late transformation of the extended oral disc used to adhere to rocks in torrent-adapted tadpoles (Fig. 6A) (Nodzenski and Inger, 1990). The late transforming oral disc may have lower sensitivity to TH due to high levels of type III deiodinases or cytosolic TH binding proteins or low TRα or type II expression levels.

Changes in tissue sensitivity may also underlie changes in sexual differentiation in desert frogs. New World spadefoot toads breed in extremely ephemeral desert pools, and rapid metamorphosis has evolved to allow tadpoles to achieve tail resorption before drying (Buchholz and Hayes, 2002). Changes in TH physiology underlie their ability to metamorphose faster than their non-desert-adapted closest relatives, where New World spadefoot toads have higher tissue content of, and faster response to, TH (Buchholz and Hayes, 2005). This endocrine evolution had consequences for the developmental timing of gonad differentiation, which is not under control of TH (Ogielska and Kotusz, 2004). Whereas gonad differentiation occurs during the larval period in spadefoot species with long larval periods, sexual differentiation occurs after metamorphosis in species with rapid metamorphosis likely because somatic metamorphosis has been accelerated relative to gonad development by increased activity of TH physiology (Fig. 6B).

4.3. Tissue response

Another means of TH-mediated evolutionary divergence is that different species may have different tissue responses to TH. Within a species, the downstream consequences of the switch from TR repression to activation is drastically different in different tissues, in that, for example, cell death and cell proliferation are simultaneously induced by TH in a tissue-dependent manner, e.g., cell death occurs in the tail, whereas cell proliferation occurs in the limbs. Presumably, tissue-specific gene regulatory cascades underlie tissue-specific responses to TH, such that different genes are induced by TH in different cell types (Berry et al., 1998a). The basis of tissue-specific responses within *Xenopus* may provide potential mechanisms for differences in the same tissue between species. A colorful example of an evolutionary change in TH-induced gene regulation cas-

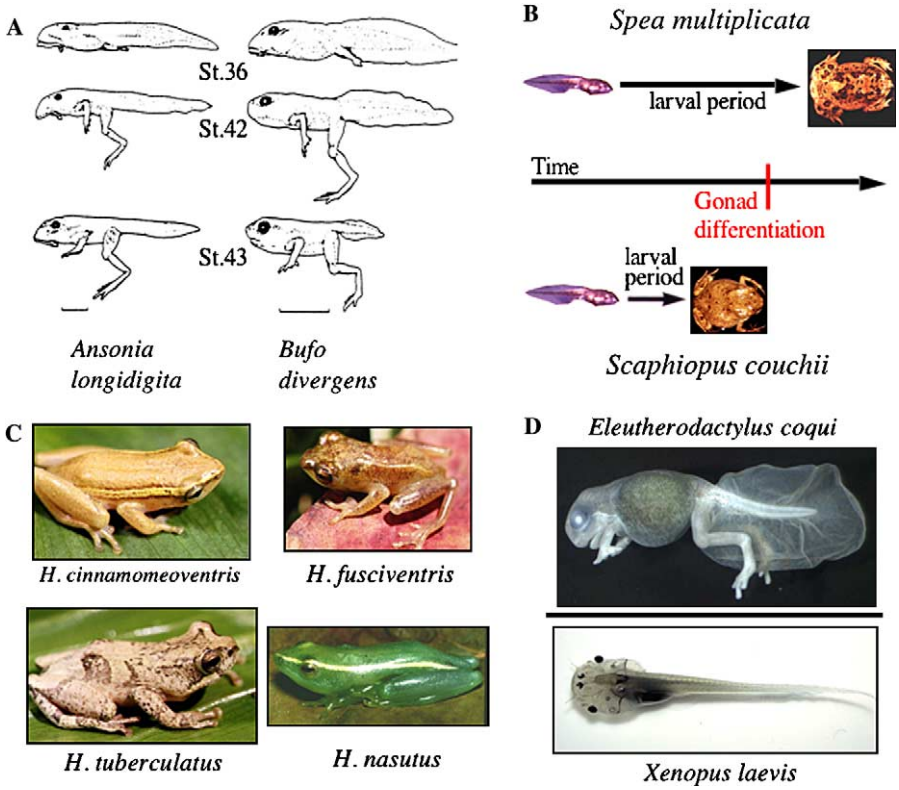

Fig. 6. Examples of endocrine evolution in frogs. (A) Tadpoles from two species in the same family, Bufonidae, grow and develop in different habitats. Tadpoles of *Ansonia longidigita* use modified larval mouth parts to adhere to rocks in fast-flowing streams, whereas tadpoles of *Bufo divergens* live in ponds and lack torrent adaptations (Nodzenski and Inger, 1990). Metamorphosis of the larval mouth parts differs between these species in that transformation of the mouth parts in *A. longidigita* is delayed until late into metamorphosis relative to *B. divergens*, presumably to allow continued adherence to rocks during metamorphosis. The hypothesized endocrine difference between these species is the presence of molecular mechanisms that decrease the effective cellular TH concentration in larval mouth in *A. longidigita*, such as increased cytosolic TH binding protein and type III deiodinase or decreased TRα. (B) Two species of spadefoot toad that differ in larval period also differ in gonad differentiation due to endocrine evolution (Buchholz and Hayes, 2005). Gonads of *Spea multiplicata* differentiate during the larval period, whereas gonads of *Scaphiopus couchii*, which has a shorter larval period, differentiate after the completion of metamorphosis. Increased TH tissue content and tissue responsivity to TH in *Sc. couchii* compared to *Sp. multiplicata* contribute to the shorter larval period of *Sc. couchii*. Because development of peripheral tissues, except the gonads, is dependent upon TH, the increased TH physiology in *Sc. couchii* caused the peripheral tissues to transform faster, leaving the gonads to develop according to their ancestral timing. (C) Endocrine evolution involves not only changes in TH production or tissue sensitivity to TH, but also changes in whether or not genes are regulated by TH, either directly or indirectly, leading to different morphological outcomes in different species. As a potential example, different species of *Hyperolius* metamorphose with different juvenile skin coloration. Replacement of larval with adult skin occurs at metamorphosis and depends on TH. These coloration differences indicate different responses in the skin to TH across species, presumably due to differential regulation of TH-response genes. (D) Some frogs, such as *Eleutherodactylus coqui*, have direct development, i.e. lack a free-living tadpole stage and hatch from the egg as a froglet (pictured embryo had egg sac removed). Direct developers require TH for proper development like all vertebrates (Callery and Elinson, 2000), but dependence of some organs to TH has been lost relative to frog species with tadpoles. Both the *E. coqui* embryo and the premetamorphic *Xenopus laevis* are shown at equivalent stages in that endogenous TH activity is low to non-existent and TH is required for further development. For example, in *X. laevis*, the limbs have not begun to differentiate because their development depends on TH, whereas the limbs are nearly fully developed in *E. coqui*. This is a striking example of a change in hormone dependence of development of the same organ in different species, and the evolutionary developmental change is completely unknown.

cades in frogs is variation juvenile skin pigmentation in African reedfrogs (Fig. 6C). Juvenile skin appears at metamorphosis and is dependent upon TH. The variety of coloration patterns bespeaks evolutionary changes across species in skin response to TH, i.e., the gene regulation cascade induced by TH in skin is different in different species.

4.4. Tissue dependence

An extreme form of change in tissue response to TH is the gain or loss of TH dependence in a tissue. Such change in tissue dependence on TH is a common occurrence in evolution. For example, birds and mammals independently evolved TH control over thermogenesis, lipogenesis, and lipolysis, whereas cold-blooded vertebrates for the most part lack this physiology (Oppenheimer et al., 1995). In development, many post-embryonic events are common among vertebrates, including neural maturation, albumin expression, hemoglobin switching, morphogenesis of limbs and lungs, chondrogenesis, and remodeling of epidermis and intestine (Tata, 1999). All these events are controlled by TH in tadpoles (Gilbert et al., 1996), and in all vertebrates studied, normal neural development is TH-dependent (Anderson et al., 2003; Denver et al., 1997;

Morreale de Escobar et al., 2004). However, the role of TH and TR in the other post-embryonic events has not been consistently conserved. Based on TR knockout mice, TR is involved in intestine development at weaning (Plateroti et al., 1999), whereas no such role for TR is expected to exist in human intestinal remodeling (Menard, 2004). In zebrafish, pectoral fin morphogenesis, chondrogenesis, and intestine development are TH dependent (Brown, 1997; Liu and Chan, 2002), though skin development apparently is not.

A striking example of changes in tissue dependence on TH within frogs is found in direct developers, where there is no free-living tadpole stage and froglets hatch out of the egg (Fig. 6D) (Elinson, 2001; Jennings and Hanken, 1998). As in *Xenopus*, adult skin formation, Meckel's cartilage proliferation, and other events are TH-regulated in *Eleutherodactylus* (Callery and Elinson, 2000). On the other hand, a long, coiled intestine is lacking in *Eleutherodactylus*, and intestine development seems to be independent of TH because hatched froglets lack a differentiated intestine which forms subsequent to hatching (Lynn, 1942). Thus, changes in tissue response to, as well as changes in dependence on, TH during development contribute to differences between direct developers and species with larval development.

The mechanisms for how tissues become dependent or independent of hormonal control are not clear. A potential class of mechanisms to change gene regulation by TR across species is through changes in the existence of a TRE in promoters of TH-regulated genes. Few such comparative studies have been done, but TH regulation of its own receptor is an exception. For example, TRβ is TH-regulated in *Xenopus* (Yaoita and Brown, 1990), *Rana catesbeiana* (Helbing et al., 1992; Schneider and Galton, 1991), zebrafish (Liu and Chan, 2002), and chicken (Forrest et al., 1990). Similarly, in rats, TRβ increases in the brain around birth coinciding with TH peak (Strait et al., 1990). In contrast, TRα, and not TRβ, is TH-regulated in turbot fish (Marchand et al., 2003). In conger eel (Kawakami et al., 2003), TRα isoforms are upregulated transiently during metamorphosis, and TRβ isoforms are upregulated at metamorphosis and remain highly expressed into adulthood. In the neotenic salamanders, TRs are not regulated by TH in the axolotl (Safi et al., 2004), and in *Necturus*, TRα but not TRβ is expressed in the intestine, liver, and gills (Safi et al., 1997). In mice (Sadow et al., 2003), TH regulation of TR is tissue-specific, where TRα is upregulated by T3 in the heart and downregulated in the liver, whereas the opposite is true for TRβ. The molecular mechanisms underlying these differences in TR regulation await characterization and comparison of TR promoters across different species.

5. Conclusion

TH plays important roles in post-embryonic development across vertebrates. Studies on amphibian metamorphosis have elucidated critical in vivo mechanisms governing the gene expression pathways regulated by TH. In particular, a dual function model for the role of TR in development has been proposed and supported by a number of in vivo studies first in *X. laevis* and then in mouse. Conservation across divergent species from fish to frog to human in the molecular mechanisms of TH signaling mechanisms suggest that changes in gene- or tissue-specific sensitivity and/or response to TH may be a significant factor in evolutionary diversity. The key to this issue is to understand mechanisms of tissue-specificity, i.e., how does the tail degenerate and the intestine remodel in response to the same TH signal. Molecularly, it is critical to understand tissue-specific gene regulation by TH within an organism, because then, we can hypothesize what is required to relieve such development of its TH dependence in the same tissue of different organisms. The first step towards determining mechanisms of TH-dependent evolutionary changes is to characterize the TH-induced gene regulation cascade in different tissues, most easily done in a model organism with a sequenced genome where hormonal control of development can be easily studied as in *Xenopus*, and then applying this knowledge towards understanding tissue-dependence on hormones in vertebrate evolution.

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