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A Dominant-Negative Thyroid Hormone Receptor Blocks Amphibian Metamorphosis by Retaining Corepressors at Target Genes

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The total dependence of amphibian metamorphosis on thyroid hormone (T_3) provides a unique vertebrate model for studying the molecular mechanism of T_3 receptor (TR) function in vivo. In vitro transcription and developmental expression studies have led to a dual function model for TR in amphibian development, i.e., TRs act as transcriptional repressors in premetamorphic tadpoles and as activators during metamorphosis. We examined molecular mechanisms of TR action in T3-induced metamorphosis by using dominant-negative receptors (dnTR) ubiquitously expressed in transgenic *Xenopus laevis*. We showed that T_3-induced activation of T_3 target genes and morphological changes are blocked in dnTR transgenic animals. By using chromatin immunoprecipitation, we show that dnTR bound to target promoters, which led to retention of corepressors and continued histone deacetylation in the presence of T_3. These results thus provide direct in vivo evidence for the first time for a molecular mechanism of altering gene expression by a dnTR. The correlation between dnTR-mediated gene repression and inhibition of metamorphosis also supports a key aspect of the dual function model for TR in development: during T_3-induced metamorphosis, TR functions as an activator via release of corepressors and promotion of histone acetylation and gene activation.

Thyroid hormone (T_3) affects a wide range of biological processes, from metabolism to development (72). The diverse effects of T_3 are generally believed to be mediated through gene regulation by T_3 receptors (TRs). In vitro and tissue culture studies have shown that T_3 activates transcription by binding to TR, which most likely heterodimerizes with RXR (9-*cis*-retinoic acid receptor) and binds to thyroid hormone response elements (TREs) in T_3 response genes. The binding of TREs by TR/RXR heterodimers is, however, independent of T_3 (9, 35, 38, 43, 62, 69), implicating a role of unliganded TR in gene regulation. Indeed, various in vitro studies have revealed that unliganded TRs repress target transcription whereas, in the presence of T_3, they enhance the transcription of these same genes (11, 23, 62, 69, 75). TRs exert these effects by recruiting TR-interacting cofactors. Many such cofactors have been isolated and characterized based on their ability to interact with TRs in the presence and/or absence of T_3 (3, 4, 29, 39, 45, 70, 72, 75).

Compared to the enormous biochemical and molecular information on TR function from in vitro and tissue culture cell studies, much less is known about TR function and associated mechanisms in development. This has been largely due to the lack of proper animal models and/or suitable methodologies for in vivo studies. Recent genetic studies in mice have provided in vivo evidence to support the critical role of TRs in mediating developmental functions of T_3. Interestingly, mice lacking TRα or TRβ or both have fewer developmental defects than mice lacking T_3 (10, 12, 13, 16, 18, 67). In addition, a major cause of resistance to thyroid hormone syndrome in humans is due to mutations in the TRβ gene, leading to the formation of dominant-negative (dn) TRβs (1, 2, 46, 72). Mice with dnTRβ, which mimics resistance to thyroid hormone syndrome in humans, and dnTRα have been analyzed at the phenotypic level but, in most cases, very little information is available on the expression of genes known to be regulated by T_3 (20, 32, 33, 61, 76).

The underlying molecular basis of the different phenotypes and differences in gene expression found in the various TR knockout and mutant animals remains unclear. Potential explanations for these differences may be found in different expression profiles and levels of different receptors, distinct isoform-dependent in vivo signaling pathways, or indirect effects through altered circulating T_3 titer. In mice with a mutation introduced into either the TRα or TRβ locus, resulting in the expression of a dnTRα or dnTRβ, a number of known T_3 response genes in different tissues were found to have different expression levels compared to wild-type mice (32, 33). However, the results from dnTRα and dnTRβ mice were quite different and could not be explained simply based on the regulation mechanisms obtained from in vitro or cell culture studies. Furthermore, it is unclear whether these genes are directly or indirectly affected by the TR knockout or transgene since no direct evidence is available to show whether TR binds to these genes and/or recruit cofactors to their promoters in the animals. In addition, other mechanisms of the regulation of T_3 target genes in these animals, such as through nongenomic mechanisms acting via cytosolic proteins (6), cannot be ruled out. Thus, to understand the developmental and pathological roles of TR, it is important to study the molecular basis by which TR mediates the effects of T_3 in various developmental processes in vertebrates.

We have been using *Xenopus laevis* metamorphosis as a model to investigate the developmental function and mechanism of gene regulation by TRs. Frog metamorphosis involves

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transformation of every organ and tissue of the tadpole and different organs and tissues undergo vastly different changes (7, 17, 52, 58, 73). Despite drastic differences across tissues, all are controlled by T_3. This dependence on T_3 makes frog metamorphosis a unique model for studying T_3 function in vertebrate development.

Here, we have generated transgenic *X. laevis* tadpoles expressing a dn form of *X. laevis* TRα (dnTR). We show that the dnTR blocks T_3-induced metamorphosis at the beginning of prometamorphosis (stage 54) (41) and that dnTR inhibits the expression of known T_3 response genes. More importantly, we used chromatin immunoprecipitation (ChIP) to show that the dnTR binds to the TREs of endogenous-T_3-response genes and retains corepressors at the target genes even when tadpoles are treated with T_3. The ChIP assay also revealed reduced histone acetylation in dnTR transgenic tadpoles treated with T_3, a finding consistent with the lack of gene activation in the presence of T_3. Thus, our results provide, for the first time in vivo, direct evidence that T3-induced development requires TRE binding by TR, release of corepressors, and consequent chromatin modification.

MATERIALS AND METHODS

Transgenesis and tadpole treatment. Transgenesis was performed as described previously (5, 14) by using the South African clawed frog (*Xenopus laevis*) (Nasco, Fort Atkinson, Wis.). The dnTR transgenesis vector pCS2G was a gift from A. Schreiber and D. Brown (51). The dn receptor has a 12-amino-acid deletion from the C terminus, preventing it from binding ligand. We also cloned the *Not*I fragment containing the crystallin promoter controlling green fluorescent protein (GFP) from CRY1/GFP3 construct (a gift from B. Grainger, University of Virginia) into the *Not*I site of pCS2G to make CGCGΔTR. This double promoter transgenesis vector regulates the expression of two genes from the same construct, GFP by the crystallin promoter and dnTR by the cytomegalovirus (CMV) promoter (14). Transgenic animals were identified by GFP expression in the eye and/or in gill and nasal regions. At stage 54, tadpoles were treated with 0, 5, or 50 nM T_3 (3,5,3′-triiodothyronine; Sigma) for 1 to 3 days at room temperature with 1 to 3 tadpoles in 500 ml. Tadpoles in treatments were not fed, and water and hormone were changed daily.

Histology and reverse transcriptase PCR (RT-PCR). For histology, tadpole intestines were fixed for 1 h at room temperature or overnight at 4°C in 4% paraformaldehyde–60% phosphate-buffered saline, cryoprotected for 2 h in 0.5 M sucrose in 60% phosphate-buffered saline, embedded in OCT medium (TissueTek), and cryosectioned at 10 μm. Sections were stained with methyl green pyronin Y (Muto, Tokyo, Japan) (25).

For RT-PCR, total RNA from tadpole organs was isolated by using Trizol reagent (Invitrogen). RT-PCRs were performed by using Superscript One-Step RT-PCR (Invitrogen) and included 0.5 μg of total RNA and two primer sets per reaction tube: one for the control rpl8 (ribosomal protein L8) and one for the gene of interest. The RT-PCR primers were designed to bind in different exons to avoid unintentional amplification of potential genomic DNA contamination. The primers (5′-3′) used were CGTGGTGCTCCTCTTGCCAAG and GACG ACCAGTACGACGAGCAG for rpl8 (57), CATCATGATTCCTGGTAA CCGA and AAATTTCCATTTTCTGCTGTGC for BMP-4 (40), GGAACTTG GAAGGTTGACAGA and GCCTCTCTTGAAAATCCTTTTTG for IFABP (55), CCTGATGCATGCAAAACT and GTTCATCCTGGAAAGCAG for ST3 (42), GGGCAGTGGACATCACCAC and GTTGACCTTGGTCTGGGCC for xhh (59), CACTTAGCAACAGGGATCAGC and CTTGTCCCAGTAGCAAT CATC for TH/bZip (15), and ATAGTTAATGCGCCCGAGGGTGGA and CTTTTCTATTCTCTCCACGCTAGC for TRβ (71). The reverse transcription reaction was carried out at 50°C for 30 min, followed by 28 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. These reaction conditions and the cycle number were based on those used previously by us and others for these genes (26, 47). Between 2 and 15 transgenic tadpoles were used for each gene-tissue combination.

ChIP assay. ChIP assay was done as described previously (50). The following antibodies were used in the ChIP assay: anti-*Xenopus* TR (68), anti-GFP (Torrey Pines Biolabs, Inc., Houston, Tex.), anti-acetylated H4 (Upstate Biotechnology, Inc., Lake Placid, N.Y.), anti-*Xenopus* N-CoR (49), and anti-*Xenopus* SMRT, which was generated by immunizing a rabbit with the polypeptide KSKKQEMI KKLSTTNRSEQE, located in a 2-kb cDNA fragment corresponding to C-terminal part, encompassing the TR-binding domain, of the *Xenopus laevis* SMRT (T. Amano and Y.-B. Shi, unpublished results).

RESULTS

Transgenic expression of a dnTR blocks T_3-induced gene regulation and metamorphosis in *X. laevis*. Using the ChIP assay, we have shown previously that TRs bind constitutively to T_3 target genes in premetamorphic tadpoles (50), supporting a causative role of the activation of T_3 target genes by T_3-bound TR in initiating amphibian metamorphosis. To investigate directly the function and the underlying molecular mechanism of gene regulation by TR in development, we used a CMV promoter in transgenesis (34) to overexpress a dnTR with GFP fused at the N terminus, which is known to inhibit T_3-induced morphological changes in very early premetamorphic tadpoles (51). When premetamorphic (stage 54) wild-type tadpoles were given a 3-day treatment of 5 nM T_3, a concentration close to the peak level of plasma T_3 during metamorphosis (36), metamorphosis was induced as expected (Fig. 1). The most noticeable external changes included the regression of gills, proliferation of Meckel's cartilage, and hind limb growth and morphogenesis (Fig. 1B and E). All of these changes were prevented in about 50% of the dnTR transgenic tadpoles treated similarly with T_3 (Fig. 1C and F). The remaining transgenic tadpoles responded to T_3 to some degree presumably due to lower transgene expression levels, as suggested by the fluorescence of the GFP fused to the dnTR in gill and nasal regions (not shown). Histological analysis of the intestine showed that T_3 treatment of wild-type tadpoles but not transgenic tadpoles expressing dnTR led to the precocious intestinal remodeling, including proliferation of the cells of the adult epithelium, connective tissue, and muscles, as well as the degeneration of the larval epithelium (Fig. 2).

To investigate whether inhibition of T_3-induced metamorphosis by transgenic dnTR expression was due to specific effects on T_3-induced gene regulation cascade, we analyzed the expression of genes known to be T_3-regulated during metamorphosis. We focused on the intestine and tail, where the dramatic gene regulation by T_3 correlates with T_3-induced morphological and cellular changes (53, 54, 65). First, we analyzed the expression of two ubiquitously regulated, direct-T_3-response genes, TRβ and TH-induced bZip-containing transcription factor (TH/bZip) (28). Because direct-T_3-response genes are known to be induced within 1 day of T_3 treatment whereas indirect-response genes require longer T_3 treatment, we investigated their regulation in wild-type and transgenic tadpoles after 1 day of T_3 treatment with either 5 nM (not shown) or 50 nM T_3 (Fig. 3). As expected, both TRβ and TH/bZip were induced by T_3 in the intestine and tail of wild-type animals (Fig. 3, compare lane 2 to 1). In transgenic tadpoles expressing dnTR, their regulation was inhibited (Fig. 3, lane 3), suggesting that dnTR directly blocks T_3 induction of these genes.

We next examined whether transgenic expression of dnTR affects gene expression associated with T_3-induced morphological changes after 3 days of treatment. We first analyzed three direct-T_3-response genes that are regulated in different organs:

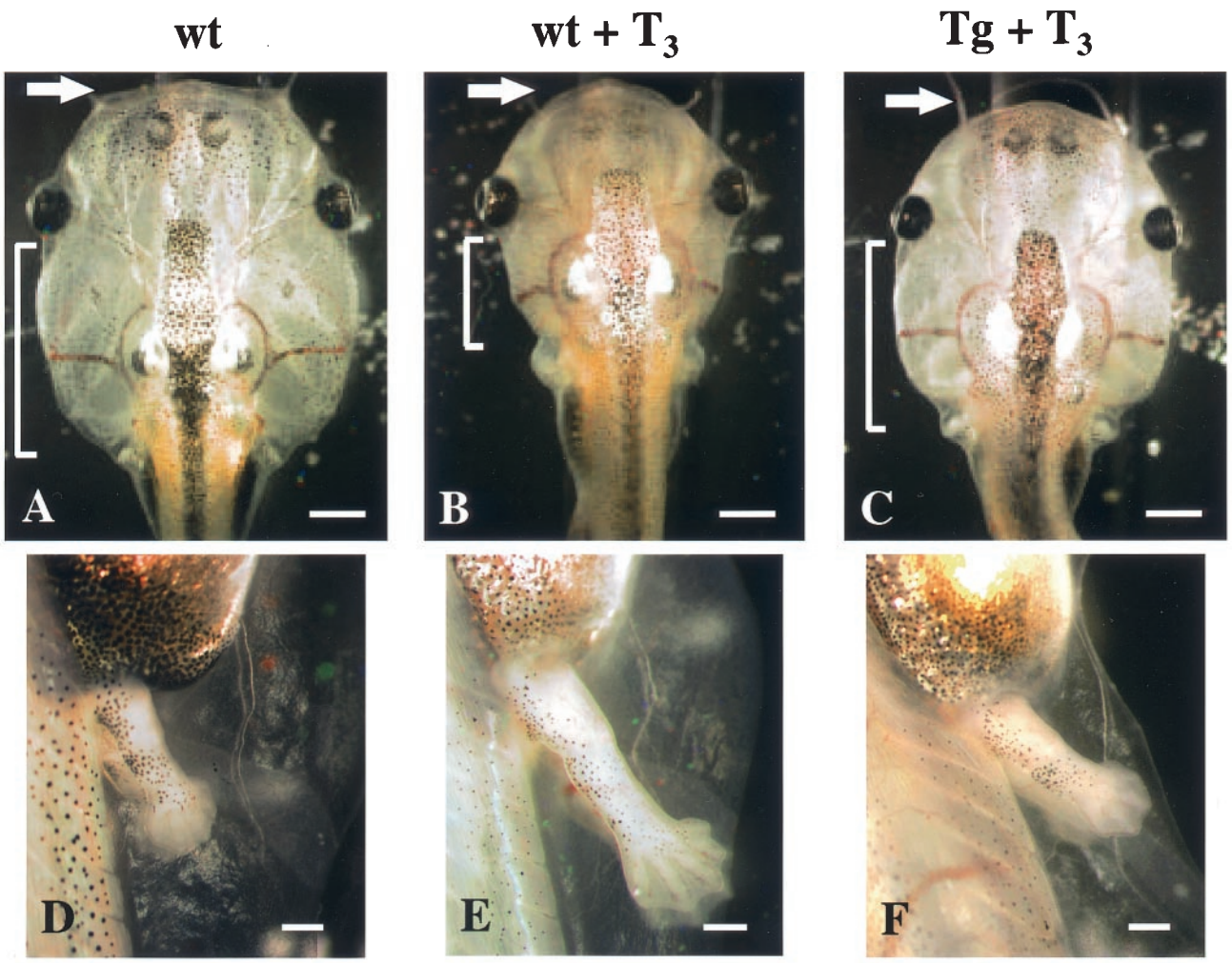

FIG. 1. Transgenic expression of dnTR inhibits T_3-induced morphological changes. Wild-type tadpoles (wt) or transgenic tadpoles expressing the dnTR under control of the CMV promoter (Tg) were treated at stage 54 with 0 or 5 nM T_3 for 3 days and then examined for changes in external morphology. Transgene expression was confirmed by observation of fluorescence in the nares from the GFP moiety at the N terminus of the dnTR fusion protein. Note that regression of gills (brackets) and proliferation of Meckel's cartilage (arrows) were induced after 3 days in wild-type tadpoles (compare panels A and B), whereas such changes did not occur in the T_3-treated transgenic animals (C). Similarly, hind limb growth and morphogenesis were induced by T_3 treatment in the wild type (compare panels D and E) but not in transgenic animals (F). In the absence of T_3, wild-type and transgenic animals are expected to be the same because endogenous TR (TRα is expressed during premetamorphosis) and dnTR function similarly without ligand, and they were indeed found to be the same during premetamorphosis (not shown). Thus, transgenic animals without T_3 treatment were not used in this and other experiments. All experiments in Fig. 1 to 6 were repeated at least three times with similar results. Bars: 2 mm (A to C) and 1 mm (D to F).

TRβ, TH/bZip, and a fibroblast-specific stromelysin 3 (ST3) (42). All three were induced in both the tail and intestine by the treatment of premetamorphic tadpoles at stage 54 with 5 nM T_3 for 3 days (Fig. 4, compare lanes 1 and 2), a treatment that induced observable morphological changes (Fig. 1 and 2). Their induction was prevented or drastically reduced in transgenic tadpoles expressing dnTR (Fig. 4, compare lane 3 to 2). In addition, several genes which are known to be regulated by T_3 mainly or exclusively in the intestine failed to respond to the T_3 treatment in the dnTR transgenic but not wild-type tadpoles (Fig. 4, compare lane 3 and 2 to 1 for the intestine samples). These included the epithelium-specific direct-T_3-response gene *Sonic hedgehog* (*xhh*) (27, 59), the connective tissue-specific late upregulated gene bone morphogenic protein 4 (BMP-4) (26), and the epithelium-specific late downregulated gene intestinal fatty acid-binding protein (IFABP) (24, 55). In particular, the regulation of the late genes BMP-4 and IFABP is associated with morphological changes in the intestine. The failure of these genes to be regulated by T_3 in the dnTR transgenic tadpoles throughout the treatment indicates that dnTR inhibits metamorphosis by blocking the regulation of both the direct and late, indirect-T_3-response genes in different organs or tissues.

The dnTR inhibits T_3-induced metamorphosis through recruitment of histone deacetylase-containing corepressor complexes even in the presence of T_3. To investigate the molecular mechanism by which dnTR blocked gene activation by T_3 in transgenic tadpoles, we first determined whether dnTR was

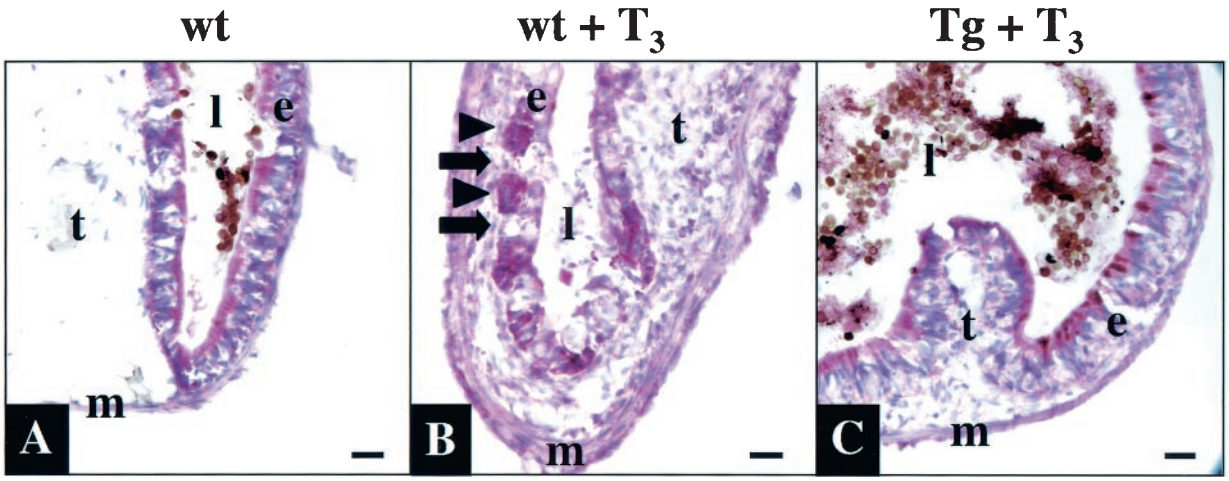

FIG. 2. Transgenic expression of dnTR inhibits T_3-induced intestinal remodeling. Wild-type (wt) or transgenic (Tg) tadpoles were treated with T_3 as in Fig. 1. Cryosections of isolated intestines were stained with methyl green pyronine Y. (A) Note that the untreated control intestine had thin muscle layers (m) and sparse connective tissue except in the typhlosole (t), which is the single epithelial fold occupying much of the lumen (l) in the larval intestine (only part of the typhlosole is shown in the image). The majority of staining was seen in the larval epithelium (e). (B) In wild-type tadpoles after 3 days of T_3 treatment, the muscle and connective-tissue layers increased in thickness largely due to active cell proliferation (56). The labeling in the larval epithelial cells decreased dramatically as these cells underwent apoptosis (arrows). Adult epithelial cells (arrowheads), whose origin remains unknown, were induced to proliferate and show intense staining. (C) All of these changes were prevented in the transgenic tadpoles treated with T_3 for 3 days. Note that the typlosoles differ in size because of intestinal remodeling and/or the sections were from different locations within the anterior intestine. The brown material visible in panels A and C is gut contents in the lumen. The results are representative of three transgenic tadpoles examined. Bar, 20 μm.

capable of binding to endogenous-T_3-response genes by competing for binding with endogenous wild-type TRs. We treated premetamorphic wild-type or transgenic tadpoles with 5 nM T_3 for 1 day and isolated nuclei from the intestines, tails, or whole animals. The nuclei were subjected to ChIP assay with polyclonal antibodies against wild-type TRs (recognizing both TRα, TRβ, and dnTR) or GFP (recognizing GFP fused to the dnTR transgene) to detect TRs bound to the endogenous-T_3-response genes TRβ and TH/bZip. As shown in Fig. 5, the TRE regions of both genes were bound by TRs constitutively in both wild-type and transgenic animals. The ChIP assay with the GFP antibody demonstrated that dnTR was also bound to endogenous TREs (Fig. 5). This is also supported by the weaker signals with the TR antibody in the transgenic animals, which was likely due to the competition for binding to the TREs by dnTR with wild-type TRs and the possible interference of the immunoprecipitation by the GFP moiety in the fusion protein dnTR.

To further understand the molecular mechanism underlying altered transcriptional regulation in the dnTR transgenic tadpoles, we investigated whether the competitive binding of TREs by dnTR in vivo altered cofactor recruitment. For this purpose, we carried out a ChIP assay to address the recruitment of corepressors and changes in histone acetylation at the

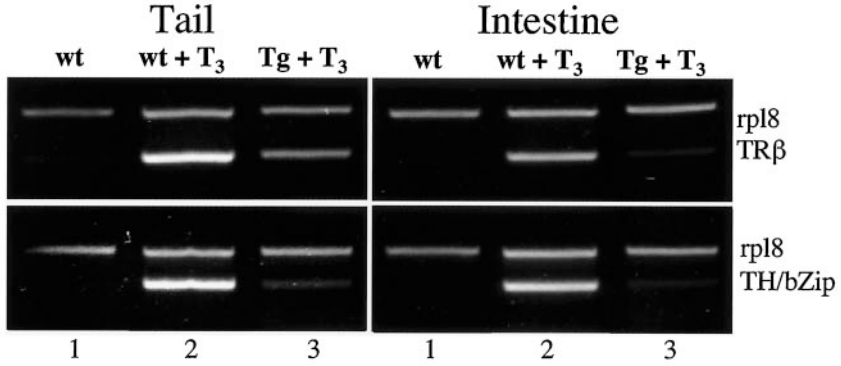

FIG. 3. The induction of direct-T_3-response genes is inhibited in transgenic animals expressing dnTR. Wild-type (lanes 1 and 2) or transgenic (lane 3) tadpoles at stage 54 were treated with 0 or 50 nM T_3 for 1 day. RT-PCR analysis was carried out for the expression of the indicated T_3-regulated genes on RNA isolated from the tail and intestine. Included in each reaction tube were a primer set for a T_3-regulated gene and one for the T_3-independent control gene rpl8 (57). The amplified DNA was analyzed by agarose gel electrophoresis. Note that TRβ and TH-induced bZip-containing transcription factor (TH/bZip) were induced both in the tails and intestines of wild-type tadpoles (compare lanes 1 and 2), as expected. Their induction was dramatically inhibited in the transgenic tadpoles expressing dnTR (lane 3).

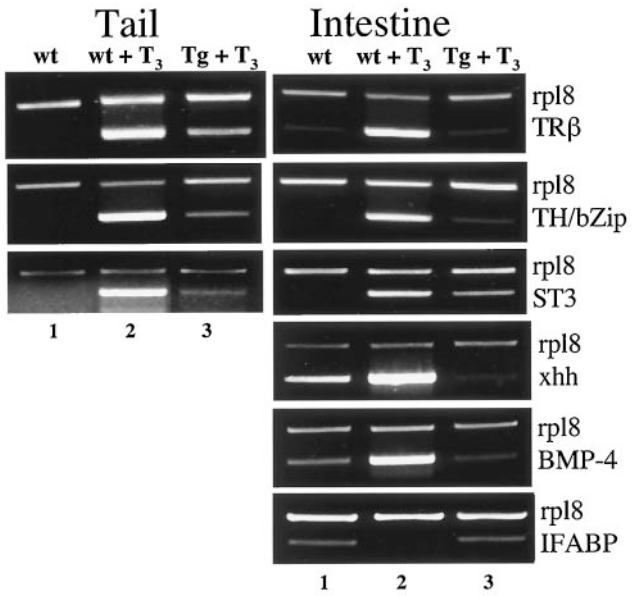

FIG. 4. Transgenic expression of dnTR inhibits T_3-induced changes in the expression of both ubiquitous and tissue-specific direct or indirect-T_3-response genes. Wild-type (lanes 1 and 2) or transgenic (lane 3) tadpoles at stage 54 were treated with 0 or 5 nM T_3 for 3 days, a treatment known to be sufficient to induce changes in the expression of both direct- and late, indirect-T_3-response genes (53). RT-PCR analysis was carried out as in Fig. 3. TRβ and TH/bZip are induced by T_3 ubiquitously, whereas ST3 is a fibroblast-specific direct-response gene. As expected, all three were induced both in the tails and intestines of wild-type tadpoles (compare lanes 1 and 2) but not in the transgenic tadpoles expressing dnTR (lane 3). In addition, the intestine-specific T_3-induced direct-response gene *Sonic hedgehog* (*xhh*), the late upregulated gene for BMP-4, and the late downregulated gene for IFABP were regulated as expected in the wild-type animals (compare lanes 1 and 2). Again, T_3 regulation of all of these genes was prevented or greatly inhibited in the transgenic tadpoles (lane 3). There are some variations in the degrees of inhibition on T_3-induced changes in gene expression among different genes and/or in different organs. This is likely due to differences between the tail and intestine and/or among the promoters of different genes.

promoters of T_3 response genes. Since the functional defect in dnTR is its inability to bird T_3, no changes were expected in transgenic tadpoles compared to the wild type in the absence of T_3. Thus, we focused on changes in T_3-treated animals. When wild-type animals were treated with T_3, the association of N-CoR to the TREs of the endogenous TRβ and TH/bZIP promoters was drastically reduced, and the acetylation levels of histone H4 was upregulated at the TRE regions in the tail and intestine, in agreement with previous observations (Fig. 6) (49, 50). In addition, by using an antibody against a peptide of the *Xenopus* corepressor SMRT, we found that it was also recruited to the TREs in the absence of T_3 and was released upon T_3 treatment in wild-type animals (Fig. 6). When transgenic animals expressing dnTR were treated with T_3, both N-CoR and SMRT were retained at the TREs (Fig. 6). Since both N-CoR and SMRT are known to form complexes with histone deacetylases (19, 30, 31, 37, 64, 66, 74), this retention of corepressors suggests that the histones at the TRE regions would be underacetylated compared to wild-type animals in the presence of T_3. Indeed, ChIP assay with anti-acetylated H4 antibody showed that the acetylation levels of H4 at the TRE regions were much lower compared to wild-type animals treated with T_3. Thus, the retention of corepressor complexes by dnTR leads to histone deacetylation at the T_3 response genes, thereby preventing their activation by T_3 treatment.

DISCUSSION

TRs are dual-function transcription factors. They bind to their target genes through TREs and repress T_3-inducible genes in the absence of T_3 and activate them when T_3 is present. They are presumed to mediate most, if not all, of the biological function of T_3 in development and organ function. Recent genetic studies in mice have provided some support for this model. However, the molecular basis for the differences in phenotypes among various TR-knockout mice, transgenic/mutant TR-knockin mice, and hypothyroid mice is unclear due to

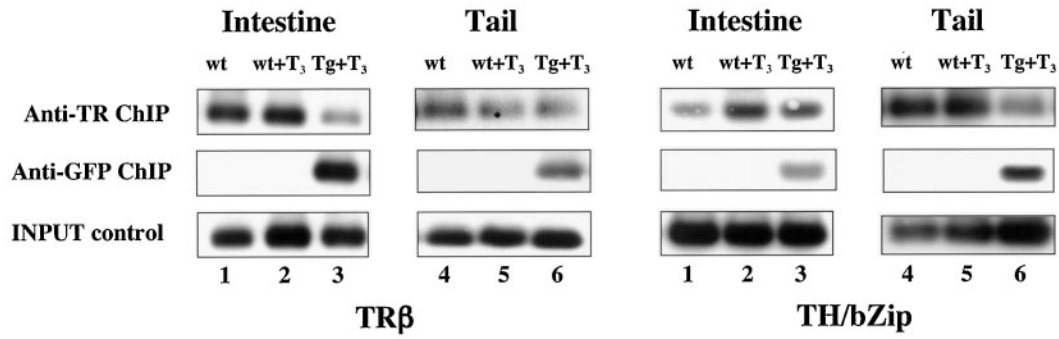

FIG. 5. The dnTR competes effectively with wild-type TRs for binding to endogenous-T_3-response genes in transgenic tadpoles. Wild-type tadpoles (wt, lanes 1 to 2) or transgenic tadpoles carrying the GFP-TR fusion gene dnTR (Tg, lanes 3) were treated with (lanes 2 to 3) or without (lanes 1) 5 nM T_3 for 1 day. Nuclei were isolated from intestine or tail and subjected to ChIP assay with the antibodies to indicated proteins for the binding of the TRs to the TRE regions of the endogenous TRβ and TH/bZip genes. Note that the ChIP assay with the GFP antibody clearly showed that the fusion protein is bound to the T_3 response genes in different organs of the transgenic animals. The ChIP assay with the TR antibody, which recognizes both TRα and TRβ, showed that TR is constitutively bound to both genes in different organs or whole animals (not shown) as expected (the weaker signal in the transgenic animals is likely due to possible interference of the immunoprecipitation by the GFP moiety in the fusion protein. Regardless of the exact cause, it does not affect the main conclusion that dnTR can compete with endogenous TR for binding to TREs). It is unclear why the T_3 treatment enhanced the binding of TR to the TH/bZip promoter in the intestines but not the tails. However, this has no impact on our conclusion regarding dnTR binding to endogenous promoters. The INPUT control shows the DNA level prior to immunoprecipitation with the antibodies.

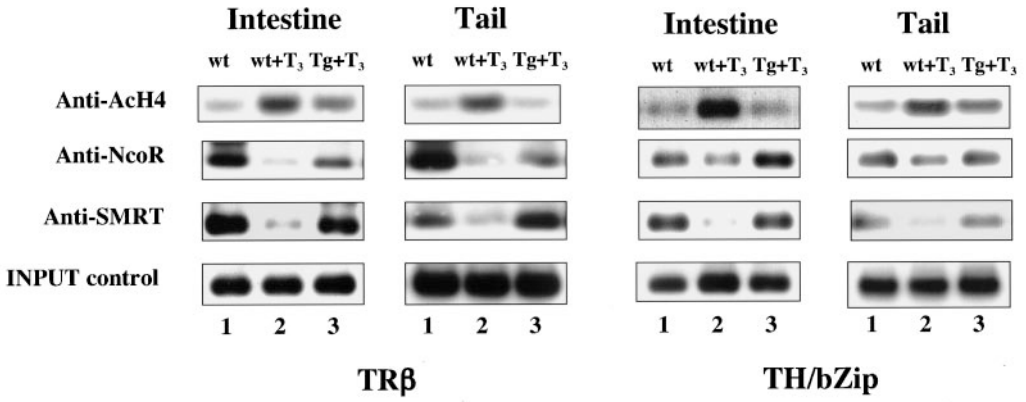

FIG. 6. The dnTR retains corepressors at the T_3 response genes even in the presence of T_3 and prevents T_3-induced histone acetylation at the target genes. Wild-type tadpoles (wt, lanes 1 and 2) or transgenic tadpoles carrying the GFP-TR fusion gene dnTR (Tg, lanes 3) were treated with (lanes 2 and 3) or without (lanes 1) 50 nM (for acetylated H4 [AcH4]) or 5 nM (for all others) T_3 for 1 day. ChIP assay was carried out as in Fig. 5 except that different antibodies were used as indicated. Note that T_3 induced the release of corepressors in wild-type animals but, in transgenic animals expressing dnTR, this release was inhibited significantly if not completely (the extent of the inhibition appeared to vary slightly in different tissues for the two genes but this does not affect the conclusion), and as a result, histone acetylation levels at the target genes in the transgenic animals were lower compared to wild-type animals even though both were treated with T_3. The INPUT control shows the DNA level prior to immunoprecipitation with the antibodies.

the lack of molecular studies on the regulation of T_3 target genes in these animals (10, 12, 13, 16, 18, 20, 32, 33, 61, 67, 76). This raises the possibility that T_3 may function through TR-independent pathways by acting through cytosolic proteins (6). In the present study we show, by using frog metamorphosis as a model, that a dnTR expressed in transgenic animals specifically inhibits the expression of endogenous-T_3-response genes to block the biological effects of T_3, i.e., the induction of metamorphosis. More importantly, we demonstrate here for the first time in developing animals that the dnTR inhibits T_3 response gene regulation by binding to TREs in the target genes, thereby retaining corepressor complexes to deacetylate histones at the target gene promoter even in the presence of T_3.

Functions of TRs during frog development. Based on the expression studies on TRs and their heterodimerization partners RXRs in *X. laevis*, we have previously proposed a dual-function model for the role of TR in frog development (48). That is, TR/RXR heterodimers function as transcriptional repressors of T_3-inducible genes in premetamorphic tadpoles to allow for animal growth and prevent premature metamorphosis and as transcriptional activators of these genes when T_3 becomes available later to initiate metamorphic changes in different tissues. Earlier work has provided several independent lines of evidence in support of this model. First, by introducing TRs and/or RXRs into developing *X. laevis* embryos, we had shown that TR/RXR heterodimers are capable of repressing endogenous T_3 resposne genes in the absence of T_3 and activating them when T_3 is present (44). Second, by transiently transfecting tail muscle cells in vivo, Tata and coworkers showed a dnTR was able to inhibit the T_3 induction of a cotransfected T_3-inducible promoter in premetamorphic tadpoles (63). Finally, we had demonstrated that TR and RXR are bound constitutively to the TREs of T_3 response genes in premetamorphic tadpoles (50), implicating a role of unliganded TR/RXR in premetamorphic tadpole development. Our data presented here showing molecular evidence in vivo is the most direct support for this model to date.

The dual function of TRs in development may not be unique to amphibian metamorphosis because recent genetic studies support, by implication without direct evidence, this model in mammals. For example, mice lacking either TRα or TRβ or both have fewer abnormalities in development and/or organ functions than are associated with hypothyroidism (12, 13, 16, 18, 21, 67). Furthermore, as in frogs, during early embryogenesis, mammalian fetuses have little or no detectible T_3 in plasma, although TRs are expressed (60). As in amphibian TR expression studies, these data imply a role for unliganded TR during early mammalian embryogenesis before zygotic synthesis of T_3, which subsequently would activate T_3-inducible genes through binding to TRs. Unfortunately, few mammalian T_3-inducible genes have been identified and analyzed during development, and no study has examined the molecular mechanism of T_3-induced transcription in vivo to determine the role of TRs in mediating the developmental effects of T_3. We directly address these issues here in vivo by using ubiquitous transgenic overexpression of a dnTR that inhibits amphibian metamorphosis. We showed that the dnTR specifically inhibited the regulation of all known T_3 response genes analyzed thus far by binding to TREs in different organs and tissues. Thus, our data provide direct in vivo molecular support for the dual-function model for TR function in development by revealing a critical role of transcriptional activation of T_3-inducible genes by T_3-bound TRs in activating morphogenic transformation in various tissues and organs.

Role of corepressors in the regulation of T_3 response genes in development. Since the cloning of TRs in 1986 (9), extensive effort has been directed toward understanding the molecular mechanisms governing transcriptional regulation by TRs, leading to the identification of many cofactors (3, 4, 29, 39, 45, 70, 72, 75). Studies in vitro and in tissue culture cells, as well as in frog oocytes, have shown that transcriptional activation by T_3-

bound TRs involves the recruitment of coactivator complexes with (e.g., SRC and p300) or without (e.g., TRAP or DRIP) histone acetyltransferase activity, in a process that also involves chromatin remodeling (3, 4, 22, 23, 29, 39, 45, 69, 70, 72, 75). In the absence of T_3, the unliganded TR repressed transcription by recruiting corepressors. The best-studied corepressors for TRs are N-CoR and SMRT. Many of the cofactors are expressed in many different cell types and tissues and thus are likely to participate in gene regulation by TRs. However, there have been few studies addressing the function of these cofactors in gene regulation by TRs in developing animals due to the lack of proper systems for molecular analysis.

Frog metamorphosis is an excellent system for analyzing TR regulation at the molecular level. Diverse changes in different organs, such as resorption of the larval gills and tail and de novo development of the limbs and adult intestine, await the cue from T_3 to initiate the metamorphic changes (7, 17, 52, 58, 73). Thus, investigation of the role of cofactors in different developmental processes is simplified by the uniform state of TR function in premetamorphosis where there is no T_3. By using the ChIP assay, we had previously shown that TR/RXR heterodimers bind TREs in premetamorphic tadpoles constitutively (50). More recently, we demonstrated recruitment of N-CoR by unliganded TRs to the target genes in premetamorphic tadpoles and its release by T_3 treatment (49). Consistent with the association of N-CoR and SMRT with histone deacetylases (19, 30, 31, 37, 64, 66, 74), T_3 treatment of premetamorphic tadpoles leads to an increase in local histone acetylation levels at the target genes upon the release of N-CoR (49). In the present study, we have extended these earlier findings by showing that *Xenopus* SMRT is also recruited by unliganded TRs in premetamorphic tadpole tissues and is released upon T_3 treatment. Thus, both N-CoR and SMRT appear to have similar function in tadpole development.

More importantly, our results for the first time demonstrate that the activation of T_3 response genes by TR is essential for amphibian metamorphosis. Transgenic expression of the dnTR specifically inhibits the regulation of both direct and indirect-T_3-response genes, and this inhibition is tightly associated with the blockage of metamorphosis. Transgenic animals in which T_3 response genes were not influenced by transgene expression (due to lower expression levels as reflected by the fluorescence of the GFP fused to the dnTR in gill and nasal regions) were able to undergo T_3-induced morphological changes (data not shown) (It is worth pointing out that, due to low expression levels of endogenous TR, which is not detectable by standard Western blot analysis [8], and the lack of an antibody that recognizes equally both the endogenous TR and transgenic dnTR, it is difficult to directly compare the levels of transgenic TR with endogenous TR. On the other hand, our phenotype and molecular analyses clearly indicated that sufficient transgenic TR was expressed in the transgenic animals to affect their development.) By ChIP assay, we have shown that this inhibition involves the binding of dnTR to TREs in T_3-inducible genes. This leads to the retention of the corepressors SMRT and N-CoR at the target genes even in the presence of T_3, a finding consistent with the inability of dnTR to bind T_3. This retention of the corepressors appears to lead in turn to histone deacetylation at the target genes, in agreement with the ability of SMRT and N-CoR to form complexes containing histone deacetylases. Thus, our data show that the release of corepressors by T_3 and subsequent histone acetylation are critical for the activation of T_3-inducible genes by TR, the first step in the gene regulation cascade responsible for T_3-dependent tissue transformation during frog metamorphosis.

In conclusion, we have shown here that during development the ligand-binding and activation function of TR is essential for amphibian metamorphosis. The total dependence of this process on T_3 and concurrent transformation of different tissues and organs have allowed us to show, for the first time in developing animals, that corepressor release and histone acetylation are important for gene activation by T_3 and subsequent morphological changes. The development of reagents for analyzing the function of coactivators should allow us in the future to test whether coactivator recruitment after corepressor release participates in this process. Further analysis in different organs and tissues with similar approaches should help to determine whether different cofactors are utilized by TR in different developmental programs, such as cell proliferation, differentiation, and apoptosis.

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