Diverse developmental programs of *Xenopus laevis* metamorphosis are inhibited by a dominant negative thyroid hormone receptor

Alexander M. Schreiber, Biswajit Das, Haochu Huang*, Nicholas Marsh-Armstrong†, and Donald D. Brown‡

Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, MD 21210

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Metamorphosis of anuran tadpoles is controlled by thyroid hormone (TH). Here we demonstrate that transgenic *Xenopus laevis* tadpoles expressing a dominant negative form of TH receptor-α are resistant to a wide variety of the metamorphic changes induced by TH. This result confirms that TH receptors mediate both early and late developmental programs of metamorphosis as diverse as growth in the brain, limb buds, nose and Meckel's cartilage, remodeling of the intestine, and death and resorption of the gills and tail.

Rising thyroid hormone (TH) levels produced by the thyroid gland of a growing tadpole orchestrate the sequential changes of metamorphosis in the majority of tadpole organs. These morphological changes range from growth and cell differentiation (limbs) to cell death and tissue resorption (tail and gills), and include the remodeling of tadpole organs into their adult forms (intestine, skin, and brain). With the discovery that the TH receptors (TRs) are transcription factors (1, 2), these varied developmental programs have been studied as complex changes in gene expression initiated by TH (3). Despite the many experiments that prove the requirement of TH in metamorphosis, there is only one demonstration to date showing conclusively that TH acts by way of TRs in a particular metamorphic program. The asymmetrical replication of the ventral retina in *Xenopus laevis* is inhibited by expression of a dominant negative form of the TR (TRDN; ref. 4).

All vertebrates studied to date, including *X. laevis* (5), have two highly conserved TR isoforms called TRα and TRβ. In *X. laevis*, tadpole TRα is constitutive and distributed widely in tissues even before the organism forms a thyroid gland (6). Because TRβ is a direct response gene of TH (6, 7), the amount of TRβ in cells increases along with the rise in endogenous TH that occurs as metamorphosis proceeds (6, 8). During premetamorphosis when the early events of tadpole development (such as limb growth and DNA replication in the brain) occur, the TH concentration and the TRβ levels are very low. TRβ and TH rise to a peak at the climax of metamorphosis when the final changes (such as gill and tail resorption and intestinal remodeling) occur. We will show that TRα is required for the precocious response of young tadpoles to exogenous TH in their rearing water. We have taken advantage of the new transgenesis method (9) to express green fluorescent protein (GFP) fused to a dominant negative form of TR-α (GFP-TRDNα) driven by two different widely expressed promoters. Metamorphic changes that are inhibited by the GFP-TRDNα include brain development, limb and Meckel's cartilage growth, intestinal remodeling, gill resorption, and death of cells in the tail including muscle.

Materials and Methods

Plasmids, Transgenesis, and 3,5,3′ Triiodothyronine (T_3) Treatment. Constructs were prepared in pCS2$^+$-based vectors and used either the cytomegalovirus (CMV) promoter/enhancer (10) or a 6-kb α2(1) mouse collagen enhancer fused to a minimal α2(1) promoter (Col; ref. 11). Constructs were made with both promoters driving either GFP alone or the described GFP-TRDNα construct (4). In this construct, GFP is fused to amino acid number 2 of TRα, and the TRα region extends to amino acid 401. The C-terminal deletion removes the ability of the TR to bind TH. To test the strength and specificity of a TRDN, different ratios of constructs encoding wild-type TRα or TRβ and TRDNα or TRDNβ were mixed with a reporter construct in which a TH response element was driving luciferase. The mixture was transfected into cultured Chinese hamster ovary (CHO) cells. These mammalian-cultured cells have low endogenous TRs (C. Thompson, personal communication). Both TRDNα and TRDNβ could suppress the increase in luciferase activity induced by TH and mediated by either TRα or TRβ. The TRDNα was several times more efficient at inhibiting the wild-type TRs than the TRDNβ, and TRα required twice the amount of either TRDN to suppress it than did TRβ. TRDNα inhibited an equal amount of TRα by about 25%. Almost complete inhibition occurred with a 5-fold excess of TRDNα. The inhibitory effect of GFP-TRDNα is only slightly weaker than the TRDNα alone.

Transgenic animals were prepared by restriction enzyme-mediated integration (REMI) nuclear transplantation (9), using minor modifications (12). Transgenic tadpoles were identified by their GFP fluorescence at day 4 of development, using a Leica (Deerfield, IL) MZ12 fluorescence stereo microscope. The distribution of tissue expression for CMV:GFP and Col:GFP was evaluated in whole mount and in cryosections of 2-week-old tadpoles. The whole-mount expression of GFP in various tissues was monitored by photographing live transgenic tadpoles, using a Leica fluorescence stereo microscope and a Spot RT camera. GFP expression was also assessed by cryosections of the head, tail, and intestine, followed by immunofluorescent detection of GFP, using a Nikon Eclipse E800 microscope and an MTI RC300 digital camera.

One-week-old transgenic tadpoles were induced for 7 days with 10 nM T_3 diluted in 0.1 $\times$ MMR (10 mM NaCl/0.2 mM KCl/0.1 mM MgCl2/0.2 mM CaCl2/0.5 mM Hepes, pH 7.5) (12). Animals transgenic for CMV:GFP and CMV:GFP-TRDNα were treated together in the same containers; animals transgenic for Col:GFP and Col:GFP-TRDNα were also treated together. Resistance to T_3 induction was assessed by the degree of change in head morphology. Tadpoles that are sensitive to T_3 fully resorb their gill arches, have a protruding lower jaw (Meckel's cartilage), a widened brain, and the nose is closer to the brain's olfactory bulb. A partially resistant tadpole has

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Abbreviations: TH, thyroid hormone; T_3, 3,5,3′-triiodothyronine; TR, TH receptor; TRDNα, dominant negative form of the TR-α; Col, promoter/enhancer for α2(1) mouse collagen; CMV, cytomegalovirus promoter; GFP, green fluorescent protein.

*Present address: Joslin Diabetes Center, 471 Brookline Avenue, Boston, MA 02215.

†Present address: Kennedy Krieger Institute, 707 North Broadway, Baltimore, MD 21205.

‡To whom reprint requests should be addressed. E-mail: brown@ciwemb.edu.

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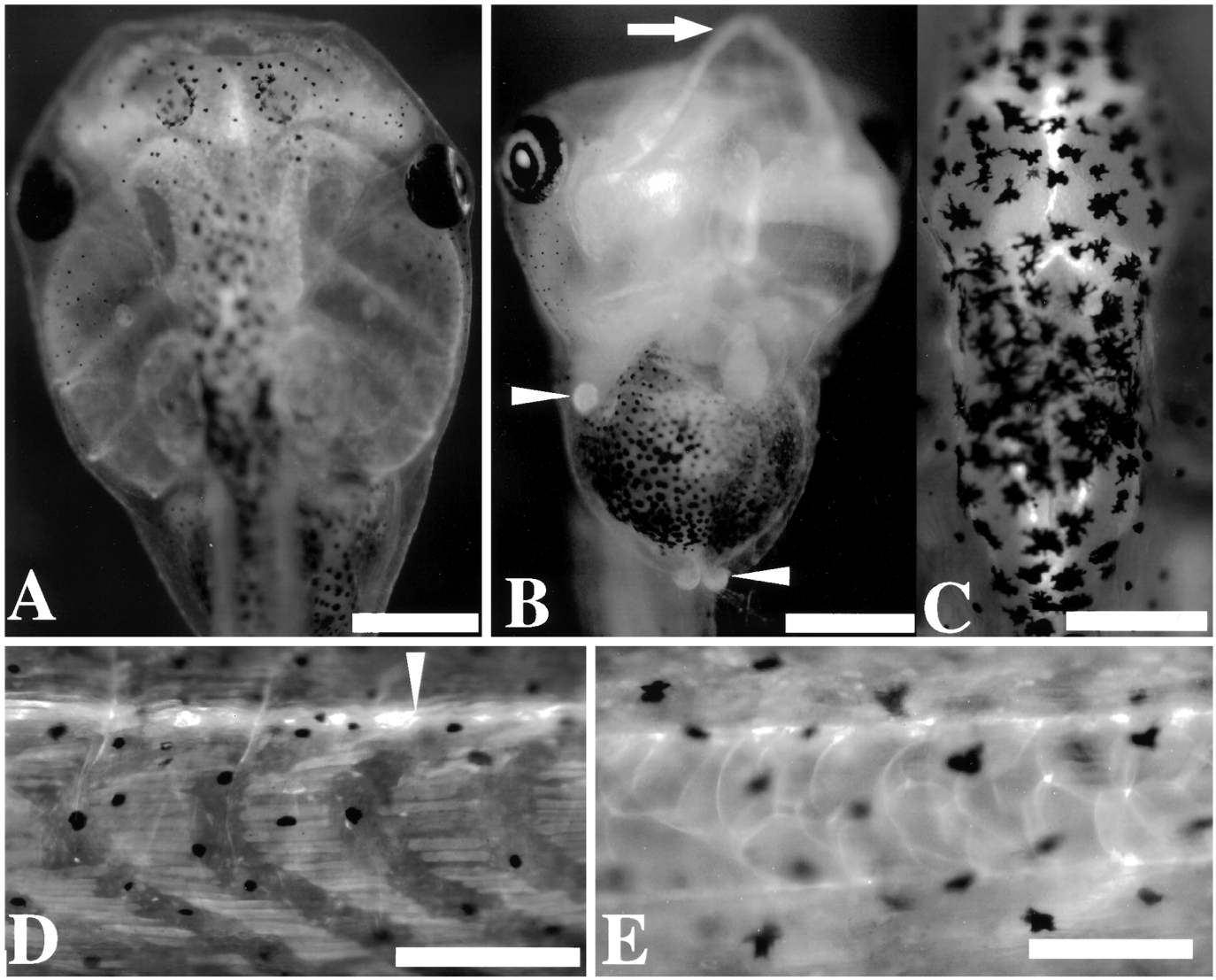

Fig. 1. Col:GFP transgenic tadpoles. (*A*) GFP expression in the head. (*B*) GFP expression in Meckel's cartilage (arrow) and limb buds (arrow heads), (*C*) brain ventricles, (*D*) tail-muscle fibers and spinal chord (arrow head), and (*E*) tail notochord. *A*, *D*, and *E* are untreated 2-week-old tadpoles; *B* and *C* are also 2 weeks old, but were treated with 10 nM T_3 during the second week. Tadpole orientations are (*A*) dorsal, (*B*) ventral, (*C*) dorsal with anterior end at top, (*D* and *E*) sagittal with anterior end left side. [Bars = 1 mm (*A* and *B*) and 500 (*C*), 250 (*D*), and 200 μm (*E*).]

incompletely resorbed gill arches, less lower jaw protrusion, and intermediate changes in its brain and nose. A strongly resistant tadpole is similar to an untreated control tadpole in all of these features.

Tail lengths (distance from tail tip to anus, mean ± SE) of some T_3-treated and untreated animals ($n = 6$) that were transgenic for either GFP-TRDNα or GFP alone were measured, and differences in length were evaluated statistically by one-way ANOVA (SuperANOVA; Abacus Concepts, Berkeley, CA). When differences were considered significant ($P < 0.05$), Fisher's probable least-squares difference (PLSD) post hoc test was performed.

Immunocytochemistry and *in Situ* Hybridization. *In situ* hybridization and hematoxylin and eosin histology were performed as described (11). GFP expression in cryosections was detected by means of a rabbit anti-GFP polyclonal Ab (Torrey Pines Biolabs, San Diego) and an Alexa Fluor 488-conjugated anti-rabbit secondary Ab (Molecular Probes). Immunocytochemistry was performed on whole-mount tadpoles after fixation in 4% paraformaldehyde in PBS for 30 min at room temperature. Tadpoles were rinsed in PBS and photo-bleached at room temperature overnight by using a ratio of 2 methanol:1 hydrogen peroxide (30%). Immunostaining of whole tadpoles consisted of extensive washes and permeabilization (at least 16 h) in PBT (1.0% Triton X-100 in PBS), blocking in 10% (vol/vol) normal goat serum in PBT for 3 h, incubation overnight at 4°C in primary Ab diluted in PBT containing 10% (vol/vol) normal goat serum, followed by an additional 1-h incubation of the primary Ab at room temperature. Samples were rinsed extensively (at least 8 h) in PBT, incubated overnight at 4°C with a fluorescently labeled secondary Ab, washed several hours in PBT, and photographed by using a Leica fluorescence stereo microscope and a Spot RT digital camera. The primary Abs were a mouse monoclonal against avian-striated muscle sarcomere myosin (MF20; Iowa Hybridoma, Iowa City, IA) diluted 1/100 and a rabbit polyclonal against human phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY) diluted 1/300. The secondary Abs were anti-mouse and anti-rabbit, Alexa Fluor 568- and 488-

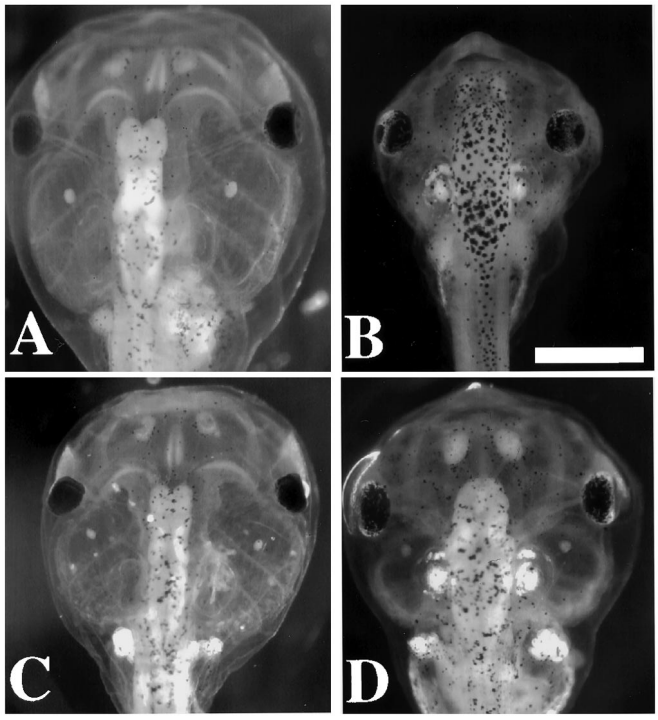

Fig. 2. Tadpoles expressing Col:GFP-TRDNα are resistant to exogenous TH. One-week-old transgenic tadpoles were treated with 10 nM T_3 for 1 week. (*A*) Col:GFP-TRDNα transgenic tadpole that had no T_3 treatment. (*B*) Col:GFP transgenic tadpole treated with T_3. (*C*) Col:GFP-TRDNα transgenic tadpole with T_3 treatment, exhibiting strong resistance to T_3. (*D*) Col:GFP-TRDNα transgenic tadpole with T_3 treatment, exhibiting partial resistance to T_3. (Bar = 1 mm.)

conjugated, respectively (Molecular Probes), that were diluted 1/400.

Results

The Col:GFP and the CMV:GFP Transgenes Are Expressed Widely. The changes that are induced in control tadpoles by the addition of 10 nM T_3 to the rearing water also occur in spontaneous metamorphosis, but in a different time sequence (12). We have used two enhancer/promoters with broad but slightly different tissue specificities to drive the GFP-TRDNα in as many cell types and tissues as possible. The GFP-TRDNα protein is localized in the nucleus of cells (data not shown), thus its direct action should be cell autonomous. The expression pattern of CMV:GFP in transgenic *X. laevis* has been described (12). The widespread expression driven by a mouse α2(1) collagen (Col) promoter has been characterized in transgenic mice (11). Fig. 1 shows the expression of Col:GFP in a 1-week-old *X. laevis* tadpole. GFP is expressed strongly in the gill arches, tail muscle, notochord, spinal cord, the cells lining the brain ventricles, limb buds, nose, and Meckel's cartilage of the lower jaw. Expression of Col:GFP in each of these tissues was confirmed by localizing GFP immunofluorescence in cryosections (data not shown). These sections also revealed that Col:GFP does not express in epithelial cells of the intestine or the skin (data not shown), which is in agreement with observations made on transgenic mouse embryos (11). In contrast, CMV:GFP is expressed strongly in epithelial cells of intestine and skin as well as gills, tail, brain ventricles, and limb buds, but weakly in Meckel's cartilage.

Tadpoles Transgenic for GFP-TRDNα Are Resistant to TH Induction. At the end of the early induction assay, 100% of tadpoles that are transgenic for Col:GFP or CMV:GFP show the same dramatic morphological changes that characterize TH-induced control tadpoles. Fig. 2 shows the typical head morphology of untreated and TH-induced control tadpoles. Treated animals have resorbed their gills completely, have protruding Meckel's cartilage, and a brain that is much wider than the untreated tadpoles with a shorter distance between the anterior end (olfactory lobe) and the nose. Fore- and hind-limb buds have developed. In contrast, the majority of tadpoles that are transgenic for GFP-TRDNα and driven by either the CMV or the Col promoters display strong resistance to these TH-induced changes (Fig. 2 and Table 1). When the phenotypes of both promoters are similar, the results of only one of them are presented below.

Table 1. Phenotypes of transgenic tadpoles expressing GFP-TRDNα after treatment with TH

	Resistance to exogenous TH		
Transgene	Strong	Partial	None
CMV:GFP	0	0	41
Col:GFP	0	0	22
CMV:GFP-TRDNα	19	17	8
Col:GFP-TRDNα	23	3	4

One-week-old tadpoles were treated with 10 nM T_3 for 1 week. Data are scored as number of individual tadpoles. Resistance to T_3 is classified based on changes in head shape (see *Materials and Methods*). Untreated tadpoles expressing each of the four transgenes appeared similar and never exhibited changes in head shape, which is characteristic of T_3 treatment (data not shown).

After 7 days of TH induction, the tail length of control treated tadpoles (3.5 $\pm$ 0.1 mm) was shortened significantly compared with untreated tadpoles (4.7 $\pm$ 0.1 mm, $P < 0.05$; Fig. 3*A*), and tail-muscle fibers were fragmented and partly resorbed (Fig. 3*C*). In contrast, the GFP-TRDNα transgene inhibited tail shortening (tail length = 4.8 $\pm$ 0.1 mm, $P < 0.05$) and muscle fragmentation (Fig. 3 *A* and *D*) compared with TH-induced controls. *Collagenase-3* (MMP-11) is a TH-responsive gene that is up-regulated in the notochord sheath at the climax of spontaneous metamorphosis (13). The expression of this gene is induced precociously by TH in the notochord sheath of a control but not in a tadpole transgenic for GFP-TRDNα (Fig. 3 *E–G*).

Some but not all of the changes in the intestine that occur during spontaneous metamorphosis can be induced by the early induction assay. The most prominent TH-induced change is proliferation and thickening of the mesenchyme (Fig. 4). The CMV:GFP-TRDNα intestines are protected broadly from TH induction.

Cell proliferation is induced by TH in spontaneous metamorphosis within Meckel's cartilage of the lower jaw, the brain ventricles, regions of the spinal cord, nose, and limb buds. The early induction assay induces proliferation in these same cells (Fig. 5). TH-induced proliferation by each of these cell types is inhibited in transgenic tadpoles that are expressing the Col:GFP-TRDNα construct. CMV:GFP expresses GFP in Meckel's cartilage more weakly than Col:GFP in transgenic tadpoles (not shown). Although transgenic tadpoles overexpressing CMV:GFP-TRDNα have a similar protected morphology as those expressing Col:GFP-TRDNα, Meckel's cartilage continues to synthesize DNA as a result of TH induction. Clearly, the morphological prominence of the lower jaw during metamorphosis is due only in part to growth of Meckel's cartilage. The other factor must be collapse of the head, which secondarily projects the lower jaw.

A summary of the distinct metamorphic programs that are demonstrably inhibited by the GFP-TRDNα driven by the two promoters is presented in Table 2.

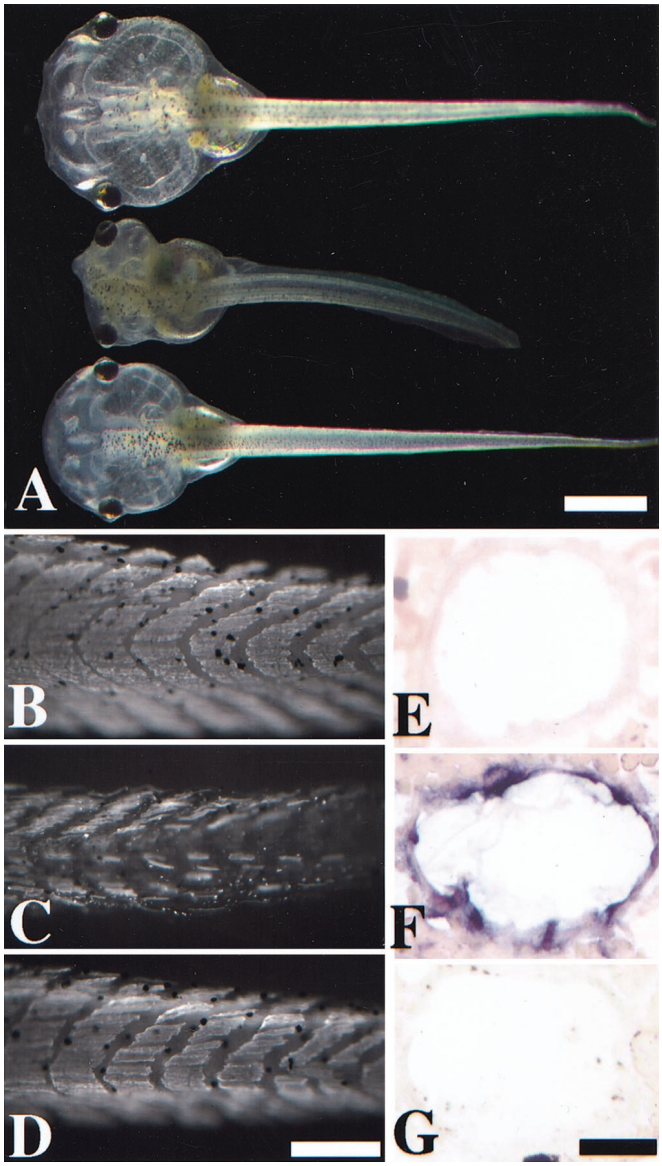

Fig. 3. Transgenic tadpoles expressing GFP-TRDNα are resistant to TH-induced tail resorption. One-week-old tadpoles were treated with 10 nM T_3 for 1 week. (*A*) Col:GFP-TRDNα, no T_3 (top); Col:GFP, with T_3 (middle); Col:GFP-TRDNα, with T_3 (bottom). (*B–D*) Tails immunofluorescently labeled in whole mount with an Ab against striated muscle myosin (MF20). (*E–G*) *In situ* hybridization of the tail notochord sheath with an antisense probe for *collagenase-3*, a TH-responsive gene. (*B* and *E*) Col:GFP-TRDNα and CMV:GFP-TRDNα, respectively, no T_3. (*C* and *F*) Col:GFP and CMV:GFP, respectively, with T_3. (*D* and *G*) Col:GFP-TRDNα and CMV:GFP-TRDNα, respectively, with T_3. [Bars = 1 mm (*A*), and 300 (*B–D*) and 40 μm (*E–G*).]

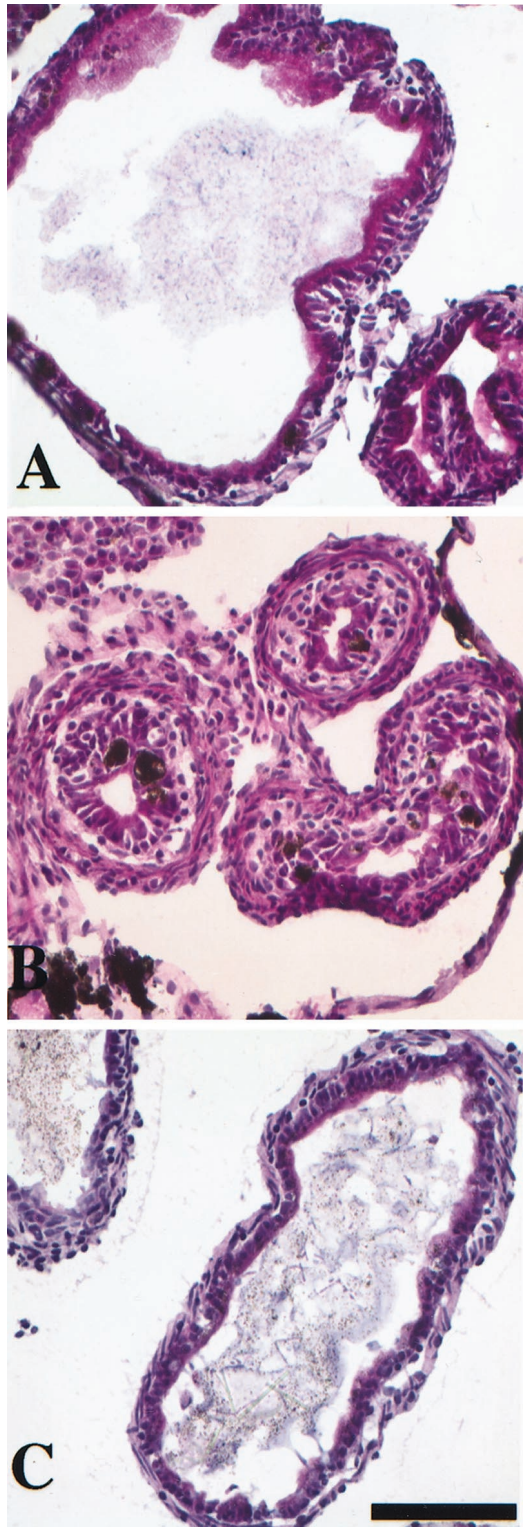

Fig. 4. Transgenic tadpoles expressing CMV:GFP-TRDNα are resistant to TH-induced intestine remodeling. One-week-old tadpoles were treated with 10 nM T_3 for 1 week. Planar sections (5 μm thick) of the intact gut were stained with hematoxylin and eosin. (*A*) CMV:GFP-TRDNα transgenic tadpole, no T_3. (*B*) CMV:GFP transgenic tadpole, with T_3. (*C*) CMV:GFP-TRDNα, with T_3. (Bar = 80 μm.)

Discussion

After the 1986 discovery in chickens (1) and mammals (2) that TRs are transcription factors, research on the action of TH focused on the hormone's influence on gene expression. The developmental programs controlled by TH in anuran metamorphosis are far more dramatic and diverse than those attributed to TH in mammals and chickens. Similarly, the extent of TH-induced gene regulation reported in tadpoles to date (14, 15) is usually manyfold greater than TH-regulated genes in mammals. Transgenic mice expressing a dominant negative TR have resulted in mild phenotypes (16). Furthermore, the individual and combined knockouts of TRα and TRβ in the mouse do not interfere with embryogenesis or fetal development (17, 18). Mice born with these mutations later display retarded growth, bone development, and reduced female fertility.

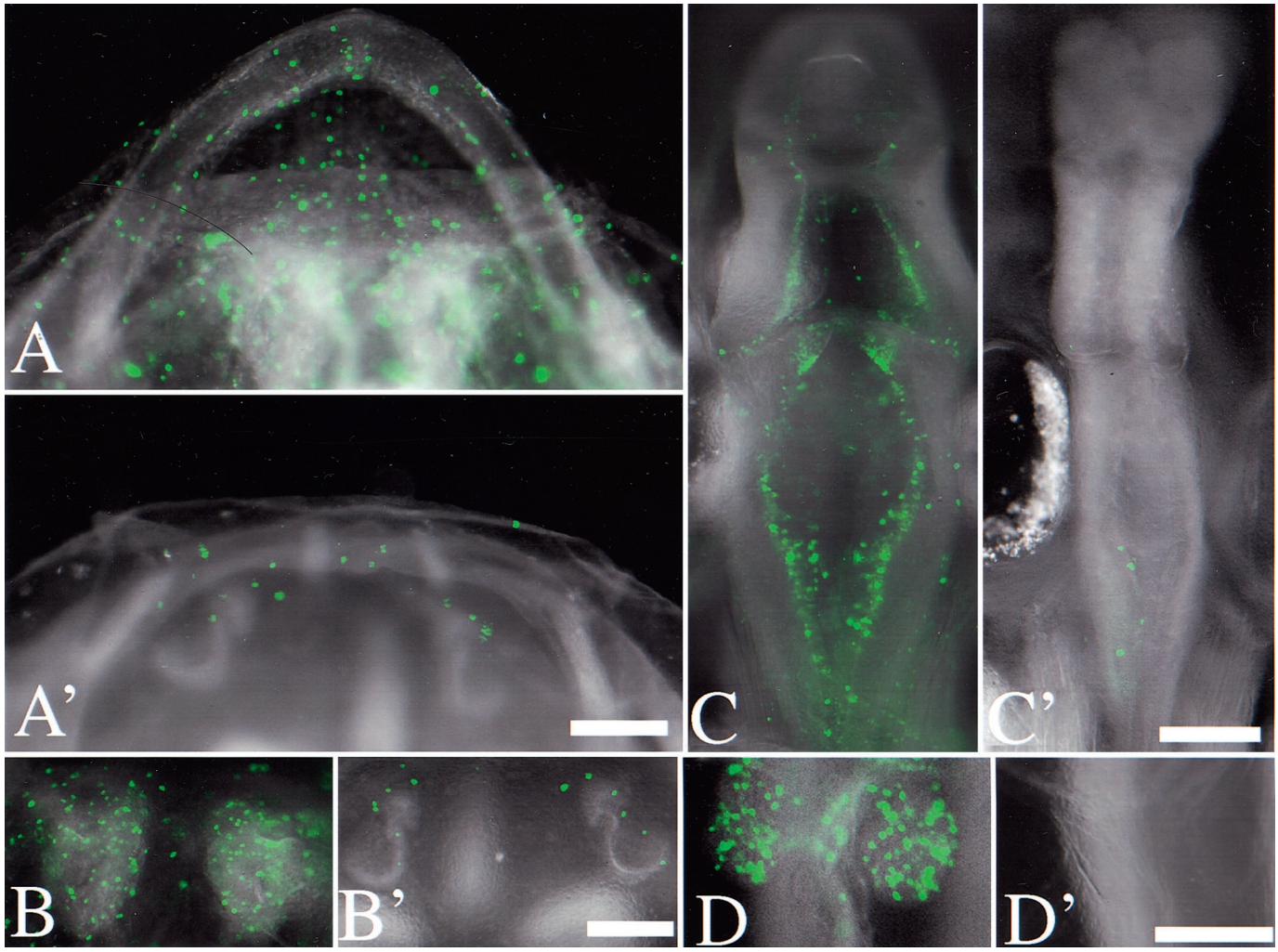

Fig. 5. Transgenic tadpoles expressing Col:GFP-TRDNα are resistant to TH-induced cell proliferation. One-week-old tadpoles were treated with 10 nM T_3 for 1 week. Proliferating cells were labeled green by whole-mount immunofluorescence with an Ab against the phospho-histone H3 mitosis marker. (*A–D*) Col:GFP control transgenic tadpole treated with T_3. (*A′–D′*) Col:GFP-TRDNα transgenic tadpole treated with T_3. Untreated controls exhibited virtually no immunoreactivity (not shown). (*A* and *A′*) Meckel's cartilage of the control; (*B* and *B′*) nose; (*C* and *C′*) ventricles of the brain; (*D* and *D′*) limb buds. (Bars = 300 μm.)

TRs have been elevated in *X. laevis* embryos by injecting *in vitro*-synthesized mRNA into fertilized eggs (19, 20). The addition of TH to these embryos causes abnormal embryonic development that mimics retinoic acid-induced teratogenesis. Expression of TRDNα or TRs with exogenous TH can cause teratogenesis (19, 20). These observations led to the suggestion (19) that TRs can repress retinoic acid-responsive genes through the retinoic acid receptor-binding elements. Overexpression of deiodinase III, the enzyme that inactivates TH, has no effect on embryogenesis (12). Our measurements of endogenous TRs detected a low level of TRα that is derived maternally; new synthesis of TRα mRNA is detectable at about stage 35 (6, 8). We believe that TH and TRs do not play a normal biological role in *X. laevis* embryogenesis. Coinjection into tadpole tail muscle of a TRDN with a reporter driven by a TH response element suppresses expression of the reporter (21); in these experiments the tail-injection method was used as a transient transfection assay. The same results were seen in *X. laevis*-cultured cells (21). However, these experiments do not address the role of endogenous TRs in metamorphosis. Although there is ample evidence that the myriad changes of anuran metamorphosis require TH, the broad involvement of TRs has not been addressed previously. To date, the only demonstration that a developmental program of metamorphosis is mediated by way of TRs was the inhibition of asymmetrical replication of the ventral retina in transgenic *X. laevis* tadpoles that were expressing TRDNα (4).

Here we show that diverse changes commonly associated with anuran metamorphosis can be inhibited by a TRDNα presenting a formal demonstration of the involvement of TRs in all of these changes. One developmental change that was not addressed in this article is the well known conversion of the tadpole epidermis to an adult germinative epithelium (22). We have demonstrated that this change is mediated by TR in *X. laevis* tadpoles transgenic for the GFP-TRDNα driven by an *X. laevis* larval keratin promoter (data not shown).

The *X. laevis* TRs. The *X. laevis* genome encodes the same two receptor isoforms (5) that have been described in other vertebrates. TRα is expressed constitutively in *X. laevis* tadpoles (6) as it is in other vertebrates (23). Its presence in tadpole cells precedes formation of the thyroid gland. TRβ is a direct-response gene of TH and its accumulation follows the endogenous TH concentration (6, 7). Experiments in cultured cells (H.H., unpublished data) show that excess TRDN derived from either TRα or TRβ can inhibit either receptor. However, there is little if any TRβ in 2-week-old tadpoles (6, 8). In spontaneous

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Table 2. Tadpole tissues resistant to TH induction by expression of GFP-TRDNα

	Transgene	
Tissue	CMV:GFP-TRDNα	Col:GFP-TRDNα
Gill arches	+	+
Tail		
Resorption	+	+
Muscle	+	+
Notochord	+	*
Intestine	+	*
Cell proliferation		
Meckel's Cartilage	−	+
Nose	−	+
Brain	+	+
Spinal chord	+	*
Limb buds	+	+

Tissue resistance to TH determined after treating 1-week-old tadpoles with 10 nM T_3 for 1 week. +, resistant; −, not resistant; *, not tested.

metamorphosis, by the time tail resorption takes place, TRβ has been up-regulated to levels that are several times higher than TRα (8). Therefore, even if later metamorphic changes require TRβ, the induction of TRβ itself depends on TRα. In the mouse, the double knockout of both TR isoforms eliminates all tight binding of T_3 in extracts, strongly supporting the idea that these two receptors are the only isoforms in the mouse (17). The possibility that *X. laevis* encodes an undiscovered TR isoform has not been ruled out. However, the TRDNα used in these experiments would be expected to inhibit only close relatives of TRα and TRβ.

Expression of the TRDNα. Two different constitutive promoters were used to ensure the broadest possible expression of the dominant negative transgene. The CMV promoter drives expression at very high levels in embryogenesis and in young tadpoles, but the expression of the transgene drops to lower levels by the climax of metamorphosis when the endogenous TR levels are elevated (12). Transient transfection studies in cultured cells suggest that TRDNα must be in excess over the wild-type receptor to inhibit TR action. We have not observed any inhibition of developmental programs during spontaneous metamorphosis by using the CMV promoter. We attribute this finding to the fact that the CMV promoter is weaker at the climax of metamorphosis. The same promoter driving expression of the enzyme deiodinase III causes tadpoles to arrest at the climax of metamorphosis (12). In contrast to the CMV promoter, the collagen promoter driving GFP-TRDNα does not cause toxicity in embryos. The transgene is expressed at a high level throughout tadpole life. When these transgenic tadpoles are allowed to undergo spontaneous metamorphosis, they die during metamorphic climax (A.M.S., unpublished data). A complete analysis of this Col:GFP-TRDNα phenotype is under investigation.

A Wide Variety of Metamorphic Programs Require TR. These experiments test whether TRs are involved in the full range of metamorphic events. We have sampled programs that are characterized by growth, death, and remodeling. Every TH-induced change that we examined is mediated by TRs. The 2-week assay is a convenient way to assay early and late metamorphic programs, because the addition of 10 nM T_3 to the rearing water for 1 week induces both early and late metamorphic events to occur simultaneously. TH induction of DNA replication precedes growth of the limb (Fig. 5*D*). An early TH-induced DNA replication in the brain and the nose also is inhibited by TRDNα (Fig. 5 *B* and *C*). Thus, both of these early events are mediated by TRs. Intestinal changes normally do not occur until the initiation of metamorphic climax but exogenous T_3 induces them precociously, and the TRDNα inhibits this response to added T_3. Two examples of cell death programs that occur late in metamorphosis at climax, gill (Fig. 2), and tail (Fig. 3) resorption are inhibited by the TRDNα. From these experiments, we demonstrate that at least the majority and probably all of the many TH-mediated changes in anuran metamorphosis begin with the hormone binding its receptor.

Some anuran tissues and organs have been shown to be autonomous in their response to TH (24). However, there is only one example of a single cell type that has been shown to respond in culture the same way that it does *in vivo*. Muscle cells have been cultured from *X. laevis* tails and have undergone cell death when induced with TH (25). The experiments reported here do not establish that any cell type which is protected from TH-induced change by the dominant negative transgene is a direct target of TH, because these promoters are expressed so widely. The extent of cell autonomy will be addressed by using cell-type-specific promoters to express the TRDN.

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