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The role of thyroid hormone in zebrafish and axolotl development

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ABSTRACT **Exogenous thyroid hormone (TH) induces premature differentiation of the zebrafish pectoral fins, which are analogous to the forelimbs of tetrapods. It accelerates the growth of the pelvic fins but not precociously. Goitrogens, which are chemical inhibitors of TH synthesis by the thyroid gland, inhibit the transition from larva to juvenile fish including the formation of scales, and pigment pattern; they stunt the growth of both pectoral and pelvic paired fins. Inhibition by goitrogens is rescued by the simultaneous addition of thyroxine. The effect of adding TH to the rearing water of the postembryonic Mexican axolotl was reinvestigated under conditions that permit continued growth and development. In addition to morphological changes that have been described, TH greatly stimulates axolotl limb growth causing the resulting larva to be proportioned as an adult in about two months. This study extends the known evolutionary relatedness of tetrapod limbs and fish fins to include the TH stimulation of salamander limb and zebrafish fin growth, and suggests that TH is required to complete the life cycle of a typical bony fish and a salamander at the same developmental stage that it controls anuran and flounder metamorphosis.**

A remarkable feature of metamorphosis in anurans and holometabolous insects is the replacement of larval by adult tissues and the large number of adult tissues and organs whose development is controlled by hormones. Even insects that develop directly with no larval stage are dependent on ecdysone to transit their life cycle (1). Adult frog organs such as the limb, intestinal tract, kidney, and skin, resemble those of other vertebrates closely yet until recently only in anurans have they been shown to require thyroid hormone (TH) to complete their differentiation (2). Recently TH-dependent metamorphosis in vertebrates was extended to include a bony fish, the flounder, which requires TH to develop beyond larval stages (3). However, the larval to juvenile transition of most bony fish and salamanders is less dramatic than that of the flounder and the frog, and is usually described as "direct development."

The zebrafish (*Danio rerio*) is a rapidly developing yet typical bony fish (Osteichthyes) that completes embryogenesis, hatches in about 3 days and then begins to feed as a larva. A pair of pectoral fins, which will differentiate as part of the larval to juvenile transition, is formed by 28 hr postfertilization (4). Smooth dorsal and ventral midline epithelial folds fuse in a seamless covering over the entire tail so that a zebrafish larva more closely resembles an amphibian tadpole than an adult fish (Fig. 1). Timing of the larval to juvenile transition that is the subject of this paper depends on the growth conditions of the larva. The transition begins several days after the larva can eat brine shrimp and is characteristic of bony fish (5, 6). Morphological changes in the zebrafish and the Mexican axolotl (*Ambystoma mexicanum*) are closely correlated with the size of the animal. When growth is slowed so is development. With the rearing conditions used here (see *Materials and Methods*), the first external signs of change to a juvenile are detectable in a 5 mm zebrafish larva about 3 weeks postfertilization (Fig. 1). The rounded end of the tail epithelium flattens as the first sign of its transformation into the typical, two pronged, homocercal adult spiny tail that characterizes bony fish. The midline dorsal and ventral unpaired fins protrude and develop, followed by the appearance of fin rays. When the larva is about 10 mm the paired pectoral fins begin to differentiate into a pair of flat, spiny fan-like paddles that splay laterally like outriggers (see Fig. 2). Several days later, when the larva is ≈13 mm, the paired pelvic fins appear on either side of the last remaining larval epithelial fold just anterior to the anus. This ventral fold resorbs as the pelvic fins grow, the striped pattern of adult fish appears, and scales form. The detailed anatomy of the paired fins and their unmistakable relationship to tetrapod limbs has been described (7). The homology between limbs and paired fins has been extended recently by the demonstration that several genes that are involved in the formation of limb buds are also actively expressed in the paired fin buds (8, 9).

Salamanders become sexually mature in a larval form, a phenomenon referred to as neoteny. The term "metamorphosis" is used in salamanders to refer to changes that occur normally or can be induced by TH in sexually mature adults and therefore are not necessary to complete the life cycle (10). Some salamanders metamorphose spontaneously. They resorb their external gills and tail fins, change their head shape, and undergo thickening of the skin. Exogenously administered TH induces these changes in facultative neotenes or paedomorphic salamanders (11) such as the axolotl that do not metamorphose normally in nature (11, 12). Obligatory neotenes such as the mud puppy, *Necturus maculosus*, do not metamorphose (according to this definition) in nature and cannot be induced to do so by administering TH (10).

The axolotl develops external gills late in the tailbud stage (day 5) followed two days later by forelimb buds (12). The embryo hatches at about day 11 and then begins to feed. At about 4 weeks postfertilization a 25-mm axolotl has developed forelimbs, but the hindlimb buds are just detectable as bean-shaped structures on either side of the posterior intestine just anterior to the anus (see Fig. 3). Over the next 3 weeks the hindlimbs elongate posteriorly, then splay laterally, develop digits, and differentiate.

This report describes the effect of exogenous TH and inhibitors of the thyroid gland on the growth and development of the zebrafish and axolotl at a stage in their life cycle comparable to anuran and flounder metamorphosis. The suggestion is that TH plays a role and may even be required for the transit from a postembryonic larva to an adult.

MATERIALS AND METHODS

Zebrafish (strain AB, ref. 14) were raised in running dechlorinated tap water ("system water") at 27°C and fed paramecia for two weeks. Then about seven larvae were placed in 2-l

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Abbreviations: TH, thyroid hormone; T_4, thyroxine; T_3, 3,5,3′-L-triiodothyronine; TU, thiourea.

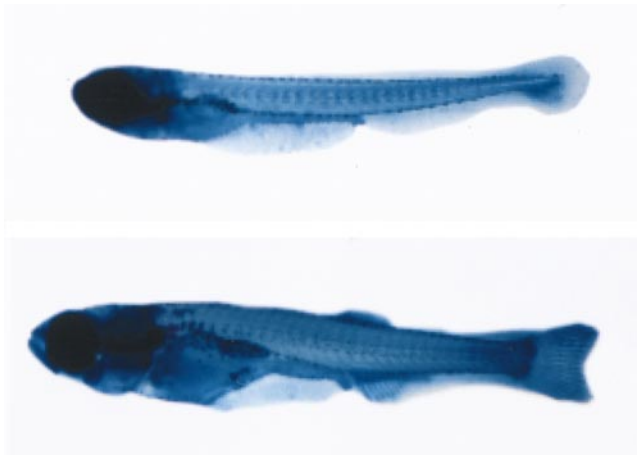

FIG. 1. (*Upper*) Two-week-old (4 mm) larva. (*Lower*) Three-week-old juvenile (6 mm) beginning unpaired fin development. Stained with alcian blue and alizarin red. Ossification has not begun. The larval paired pectoral fins are not visible in this side view.

tanks containing thyroid gland inhibitors and/or TH. The medium was changed 3 times a week. Zebrafish were fed brine shrimp for the remainder of the experiment. Results were the same if inhibitors or TH were added as early as the fourth day postfertilization. Under these conditions about five larvae were raised in 100 ml beakers of system water containing the appropriate hormone and/or thyroid gland inhibitor with daily changes. They were diluted to the larger volume when they were capable of eating brine shrimp.

Wild-type axolotl embryos were purchased from the axolotl facility at the University of Indiana, and raised in system water (500 ml per animal) at room temperature. Feeding with brine shrimp began at day 14 postfertilization. When the larvae were about 3 cm (1 month old) their diet was changed to Tubifex and their volume was increased to 1 l per animal.

Final concentrations of hormones added to the system water of either zebrafish or axolotl, unless otherwise indicated, were 5 nM 3,5,3′-L-triidothyronine (T_3) or 30 nM thyroxine (T_4). The lowest dose tried, 10 nM T_4, also stimulated limb/fin development. However, 30 nM T_4 was used routinely because it was remarkably nontoxic. Zebrafish grown in 0.5 mM or higher methimazole died at ≈12 mm having formed their unpaired fins but before differentiation of the paired pectoral fins (see Fig. 5). A concentration of 0.3 mM methimazole was the highest nontoxic dose. Even 0.1 mM methimazole arrested the development of axolotl. The animals stopped feeding and developed limb abnormalities. Zebrafish and axolotl were raised in 0.025% and 0.05% $KClO_4$, respectively. The higher dose was toxic for zebrafish. Concentrations of 0.005% and 0.003%, respectively, for 6-n-propyl-2-thiouracil and thiourea (TU), were not toxic for either animal, but they failed to inhibit [^{125}]I uptake (Fig. 4). Inhibitors and thyroid hormone were purchased from Sigma.

Iodide uptake was carried out in system water. A stock solution of [^{125}I]-NaI (NEZ-033A from DuPont/NEN) was diluted to 1 mc/ml in 0.01 mM NaOH and stored at 4°C. Zebrafish were incubated in 1–2 uc/ml of radioactive NaI for 2–6 hr at room temperature in 5–15 ml of system water, depending on their size. Axolotl were incubated overnight in the same concentration (30 ml per animal). Following their exposure to radioiodide, animals were cooled in an ice bath and fixed in 10% formaldehyde in PBS overnight. The radioactive animals were washed with many changes of PBS until there was no more soluble radioactivity released (at least 48 hr for zebrafish and twice that long for axolotl). The animals were placed on Schleicher & Schuell medium thick gel blot paper, covered with Saran wrap, dried in a gel drier, and autoradiographed. Incorporation of radioactive iodine in the thyroid gland was quantified with a PhosphorImager.

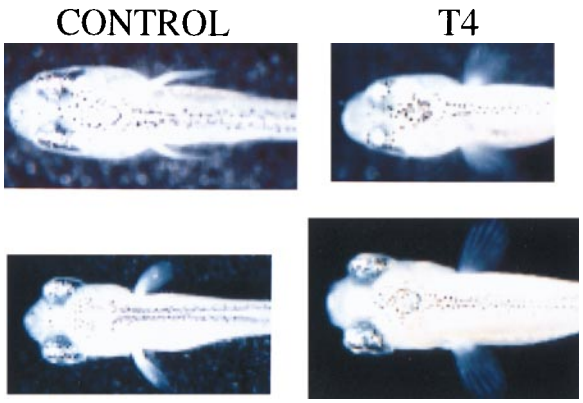

FIG. 2. Premature development of the paired pectoral fins induced by exogenous T_4. Larvae were treated continuously with 30 nM T_4 beginning 11 days postfertilization. *Upper,* the two animals (length, 6.5 mm) were fixed 23 days postfertilization. *Lower*, the two animals (8.5 mm) were fixed several days later.

Larva and adults were stained with alcian blue and then alizarin red after fixation in 10% formaldehyde in PBS for 24 hr.

RESULTS

The Effect of Exogenous TH on the Development of the Zebrafish and Axolotl. TH added to the water of zebrafish larvae throughout the first 2 weeks of larval development has no visible effect on larval development until the juvenile transition begins. T_4 treated larvae undergo premature differentiation of their pectoral fins so that they develop as early as the appearance of the unpaired fins (Fig. 2). Exogenous T_3 causes the same precocious stimulation of pectoral fin development as T_4, but it is more toxic and slows subsequent growth and development. The pectoral fins of T_4-treated larvae grow larger than control fins at the same stage. Development of the unpaired fins, resorption of the epithelial folds, and the appearance of paired pelvic fins is not accelerated by exogenous TH. However, the paired pelvic fins grow more rapidly in TH-treated animals than controls.

The continuous addition of T_4 to the water of axolotls beginning 14 days postfertilization induces noticeable resorption of the gills by the 28th day (two weeks after the addition of T_4) as described (15). The first morphological difference between T_4-treated and control axolotl forelimbs occurs after the forelimb has begun digit formation (Fig. 3). The hindlimb buds appear at the same time in T_4 treated and control animals, but when they begin to splay laterally and develop digits the T_4-treated limbs grow much more rapidly. T_3 added to the rearing water also enhanced the growth of axolotl limbs, but its effect was not followed beyond a month because of toxicity. Histological sections (not shown) reveal that the T_4-induced limbs have much greater muscle mass and long bone diameter than control limbs. Initially the larval gills do shrink, but T_4 added to the rearing water does not induce their complete resorption (as is reported to happen when the larvae are injected with TH, ref. 15). Instead, the large flowing larval gills change to resemble adult gills and then grow proportionately with the body length. Under these conditions the dorsal fin does not resorb and skin pigmentation changes prematurely in TH-treated animals to an adult pattern. Although the T_4 treated animals resemble miniature adults in 2 months, they do not have mature gonads.

Thyroid Gland Function and Inhibition. Although there have been measurements of whole body levels of both T_3 and

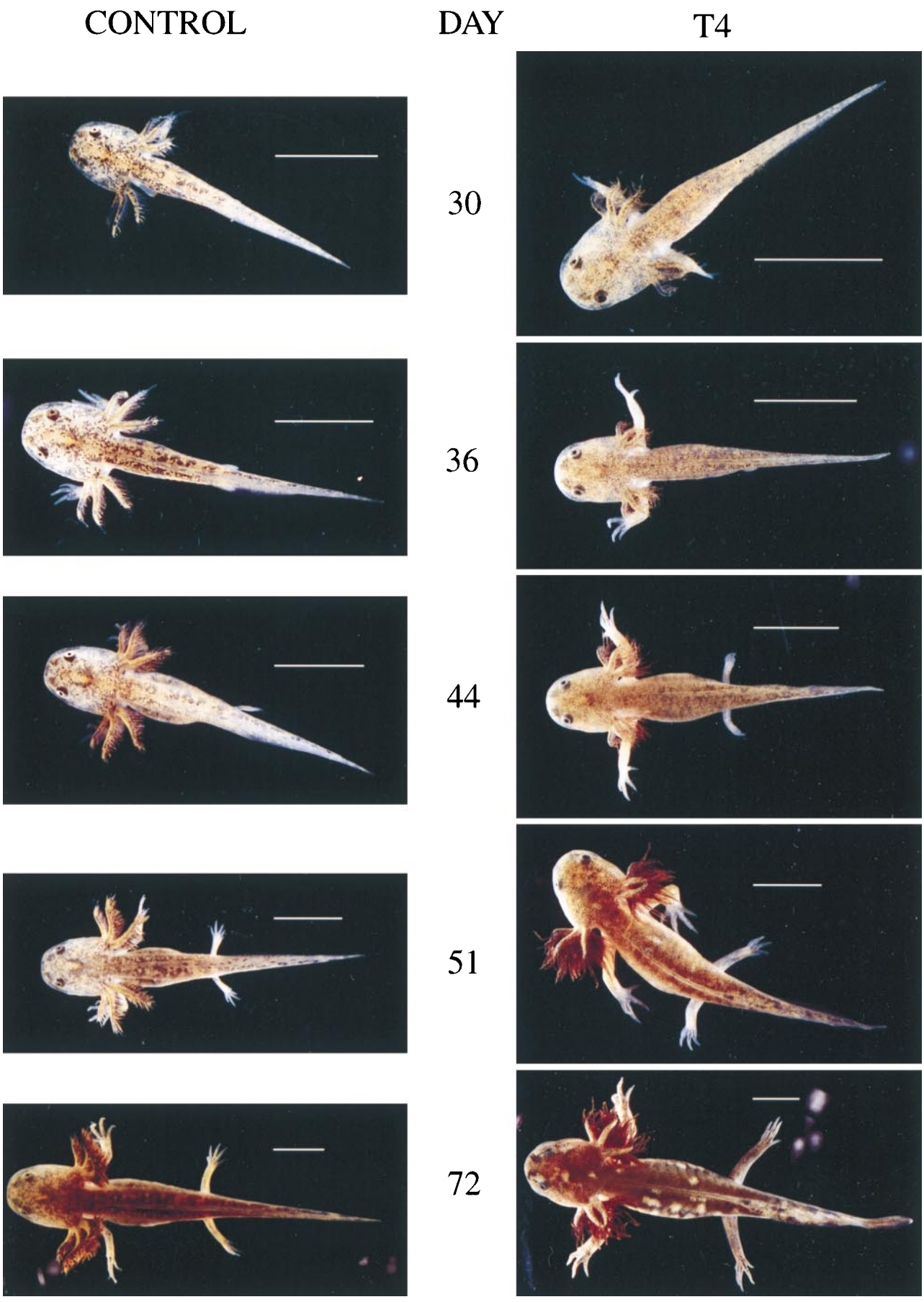

FIG. 3. The effect of thyroxine on the early larval development of the axolotl. The same control and 30 nM T_4-treated (TH) sibling animals were photographed at the days postfertilization noted. T_4 was added from day 14. (Bar = 1 cm.)

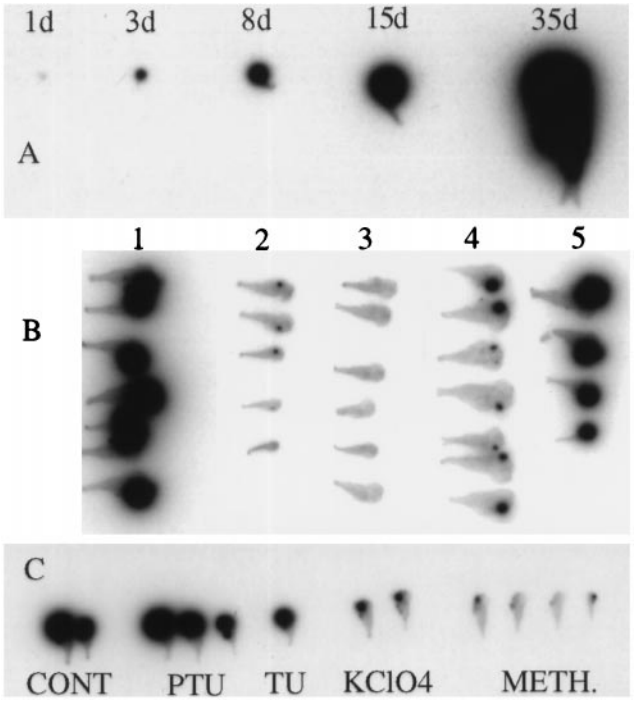

FIG. 4. Thyroid gland function assessed by [^{125}]I uptake. (*A*) Developmental series in days postfertilization. (*B*) Goitrogens and/or 20nM T_4 added on day 4 and radioiodide uptake on day 24. Lanes: 1, control; 2, 1 mM methimazole; 3, 1 mM methimazole and 20 nM T_4; 4, 20 nM T_4; and 5, 1 mM methimazole until day 12 then control until day 24. (*C*) Four different goitrogens were added at day 12 postfertilization; radioiodide uptake was assayed on day 33. Inhibitor concentrations were: 0.005% 6-n-propyl-2-thiouracil, 0.003% TU, 0.05% $KClO_4$, and 0.3 mM methimazole.

T_4 throughout larval development and metamorphosis of the flounder (16, 17), there are no such reports for the zebrafish. I chose to assess thyroid gland function by its ability to concentrate radioiodide presumably as iodinated thyroglobulin. Although levels of circulating hormone, and most importantly, the concentration of TH that reaches its receptors in cell nuclei is regulated by many steps, the uptake of radioiodide is a sensitive monitor of the thyroid gland's ability to synthesize hormone. The reduction of radioiodide uptake by inhibitors of thyroid gland function (goitrogens) estimates the effectiveness of their inhibition. Zebrafish at various stages of development were incubated with radioactive [^{125}I]-NaI, fixed and washed, the animals dried on filter paper, and then radioautographed. The single zebrafish thyroid gland fixes radioiodide for the first time at the third day of development after fertilization, and uptake increases throughout the larval and transitional periods to the juvenile stage (Fig. 4*A*). The pituitary has been implicated in the control of fish thyroid gland function (18) just as it has in frogs and other vertebrates. Exogenous TH depresses the uptake of radioiodide by the zebrafish thyroid gland (Fig. 4*B*) as it does in *Xenopus laevis* (19) presumably by shutting down thyrotropin synthesis by the pituitary. The other half of the negative feedback loop between the pituitary and the thyroid in zebrafish, reflected by goiter formation in the thyroid gland following long-term interruption of thyroid hormone synthesis, was not observed in the zebrafish or the axolotl. However, this might require a longer exposure to an inhibitor of TH synthesis than the several weeks used in these experiments.

Typical of amphibians, the axolotl has paired thyroid glands, but the uptake of radioiodide is much less active and variable in the time of its onset in axolotls than in zebrafish or *X. laevis*. Two sibling animals exactly the same size and at the same developmental stage took up very different amounts of radioiodide (data not shown). Variability of serum TH levels has been reported for another salamander (20). The earliest unequivocal incorporation of radioiodide into the pair of axolotl thyroid gland was 35–40 days postfertilization when the larvae were ≈3 cm. This is about the developmental stage when exogenous TH can first be shown to affect limb growth and gill resorption. At this time their forelimbs have completed digit formation, and their hind limb buds have begun to grow. Radioiodide uptake by the thyroid gland increases as the axolotl larva grows suggesting that there is at least a low circulating level of TH during the larval period (data not shown).

Four goitrogens that inhibit frog metamorphosis were tested for their effect on zebrafish and axolotl thyroid gland function and larval development. Methimazole and $KClO_4$ were the most effective of these inhibitors (Fig. 4 *B* and *C*). We have raised *X. laevis* tadpoles for months in 1 mM methimazole (unpublished data). This concentration markedly arrests tadpole metamorphosis, and the animals grow and develop large goiters. Doses of methimazole >0.5 mM were unpredictable in their toxicity to zebrafish larvae. Surviving larvae did not grow longer than 13 mm. Although they developed unpaired fins at the same time as controls, their paired fins did not differentiate (Fig. 5 *A* and *B*). Radioiodide uptake in these animals was inhibited >98% throughout their development (Fig. 4*B*). Animals grown in the highest nontoxic concentration (0.3 mM) of methimazole were retarded but not arrested in their development. Radioiodide uptake in these animals was inhibited ≈95%. Notable development inhibitions include stunted pectoral and pelvic fins and retarded resorption of the ventral epithelial fold. Adult pigmentation and scale formation were delayed. However, with time these animals escaped this inhibition and continued their development. The highest non toxic concentration of the goitrogen $KClO_4$ (0.05%) that inhibited radioiodide uptake by zebrafish ≈90% (Fig. 4*C*), caused the same phenotype as methimazole (Fig. 5). These animals progressed to ≈17 mm, had stunted pectoral and pelvic fins, and the same scale and pigment inhibition that was seen with methimazole. Animals treated with T_4 simultaneously with either of these inhibitors developed their paired fins and continued their growth. One group of animals were grown in the presence of $KClO_4$ past the time when controls had differentiated their paired fins. Then T4 was added along with the inhibitor; these animals grew more rapidly and developed even larger paired fins than controls (Fig. 5).

Prolonged growth of axolotls with goitrogens gave inconclusive results. Methimazole efficiently inhibited radioiodide uptake but was toxic. Doses as low as 0.1 mM arrested growth and caused abnormal development of the limbs. Unlike the zebrafish the effect of methimazole on axolotl growth and development was not reversed by simultaneous addition of T_4. Axolotls grown for as long as 3 months in the presence of 0.05% $KClO_4$ were indistinguishable from controls. This concentration of $KClO_4$ inhibits radioiodide uptake by the thyroid gland by ≈60% as did 0.005% 6-n-propyl-2-thiouracil. Even the combination of these two inhibitors had no effect on axolotl development.

DISCUSSION

Zebrafish. Following embryogenesis bony fish develop through a larval stage before their transition to adults (5, 21). Although the role of thyroid hormone (TH) in the larval to adult change had been suspected for many years (5, 6), only recently was TH shown to be required for metamorphosis in a bony fish, the flounder. The symmetrical larva rotates one eye across the skull so that both eyes face up on the same side of the asymmetric adult when it settles on the sea bottom (22). Inui and Miwa (3) have induced these changes prematurely in

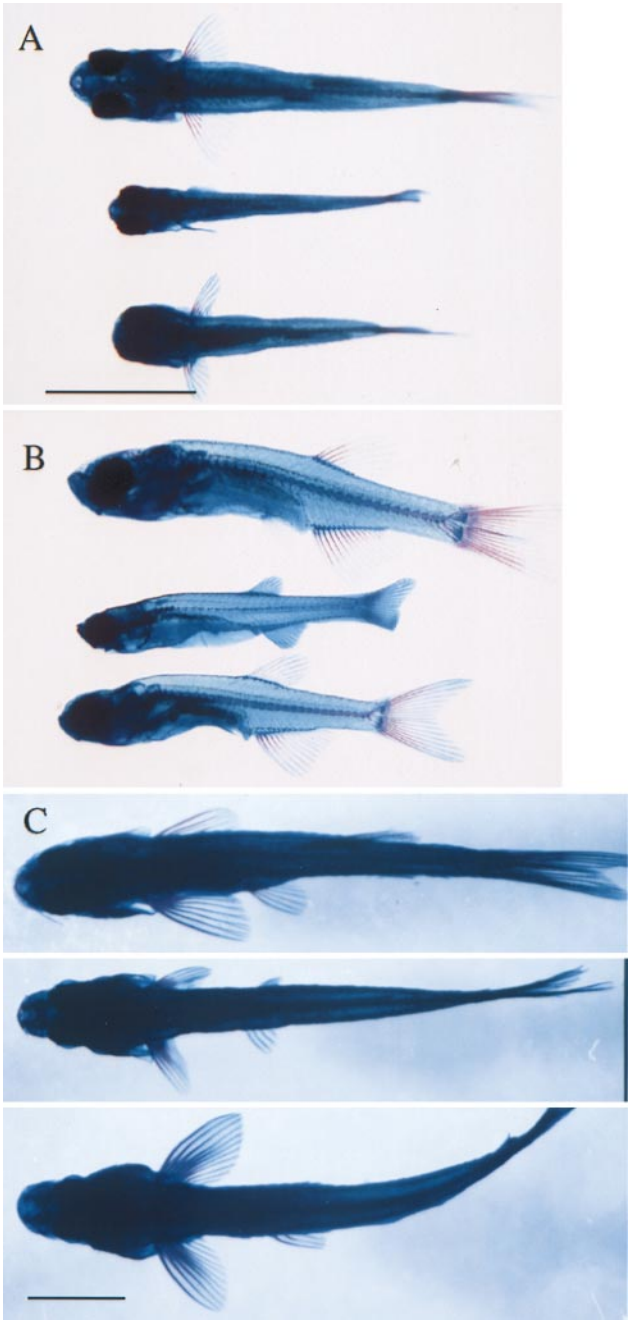

FIG. 5. The effect of goitrogens on zebrafish metamorphosis and rescue by T_4. (*A*) Dorsal and (*B*) side views of sibling fish fixed and stained with alcian/alizarin on day 33 postfertilization: (*Top*) control; (*Middle*) treated continuously from day 4 with 1 mM methimazole; (*Bottom*) treated similarly with methimazole but rescued by the addition of 30 nM T_4 beginning at day 11. (*C*) Sibling fish were fixed and stained with alician/alizarin at day 65 postfertilization: (*Top*) control; (*Middle*) treated continuously from day 14 with 0.025% $KClO_4$; (*Bottom*) treated similarly with $KClO_4$ but rescued by the addition of 30 nM T_4 beginning at day 56. (Bar = 3 mm.)

the flounder by exogenous TH and inhibited the spontaneous transformation with TU, a member of the thionamide family of compounds that block iodination of thyroglobulin in the thyroid gland. As is the case for anuran tadpoles (2), the TH level in larval flounders reaches a maximum at the climax of metamorphosis when the major changes are occurring (16). The other fish that has been studied in detail is the lamprey, an agnathan vertebrate. At metamorphosis the lamprey forms eyes, changes its mouth structure, and the dorsal fin enlarges (21). TH is reported to have a paradoxical role (23). The hormone concentration rises as larval life proceeds just as it does in anuran tadpoles, but then TH drops to low levels at the initiation of metamorphosis. The goitrogen, $KClO_4$, induces lamprey metamorphosis prematurely (24).

The circulating level of TH was not measured in the zebrafish, but the thyroid gland becomes functional at the onset of feeding, 3 days postfertilization, and then increases in activity throughout larval life and the juvenile transition (Fig. 4*A*). Exogenously added TH induces differentiation of the pectoral fins precociously (Fig. 2). Although it accelerates the growth of the paired pelvic fins, it does not induce their differentiation prematurely. Inhibition of the thyroid gland's function with goitrogens had no effect on larval development but inhibited the larval to juvenile transition in particular the growth and differentiation of the paired fins, and the differentiation of skin including pigment pattern and scales. $KClO_4$ produced the same phenotype as methimazole that is strong evidence that the goitrogen affect is caused by TH deprivation rather than nonspecific toxicity. Both methimazole and $KClO_4$ phenotypes are reversed by simultaneous addition of T_4 (Fig. 5). At a time when the goitrogen-inhibited animals arrest in growth they have undergone several obvious changes en route to adulthood that appear not to be controlled by TH. These include a change to the fish-like body shape, formation and development of the three unpaired fins, and beginning ossification of the skull and spine. One of the more drastically remodeled organs in the anuran tadpole is the intestine (2). Larval zebrafish continue feeding throughout their transition to juveniles, and the intestine grows in diameter and length. This folding and lengthening of the intestine is inhibited by methimazole and $KClO_4$ that may be a result of the general growth arrest. Although the zebrafish larval to adult transition is less dramatic than that of the flounder or the frog it occurs at a similar interval of the life cycle. Because the zebrafish life cycle is typical of many bony fish, the TH-dependent developmental transition could be a general property of bony fish.

The Axolotl. The effect of TH on axolotl development has been studied extensively (10). The reason for its reinvestigation here was twofold: the finding that TH influences fin development in zebrafish as it does limb development in the frog, and the observation that T_4 added to the water is remarkably nontoxic. The term "metamorphosis" applied to salamanders has always referred to spontaneous or TH-induced changes that occur in sexually mature adults long after limb development is completed. However, Prahlad and DeLanney (15) demonstrated that TH injected into axolotls as young as 9 days after fertilization can induce some of the same changes that had long been known to occur in adults, namely gill resorption, skin change and tail fin loss. Their failure to observe the limb changes noted here probably was due to the toxicity of the injection route of the hormone. When T_4 is added to the water over a period of weeks, the gills first shrink but then their morphology changes so that they resemble adult gills, and they increase in size proportionately with the animal's growth. The result is an animal that looks remarkably like a miniature adult (Fig. 3). Attempts to inhibit axolotl development with goitrogens were inconclusive. Methimazole, the most efficient inhibitor of thyroid gland function, was toxic even at concentrations as low as 0.1 mM. Although high concentrations of $KClO_4$, 6-n-propyl-2-thiouracil or TU did not interfere with limb development, neither did they inhibit thyroid gland function by more than about 60%. Axolotl larvae were reported to have high serum TH early in development and again at metamorphosis (25). However, another study only found significant endogenous TH at metamorphosis (20). Radioiodide uptake gave no suggestion of a functional thyroid gland before ≈30 days postfertilization but then radioiodide uptake increased with development.

There is a great diversity of amphibian life cycles. Direct developing anurans, that have no tadpole stage, are resistant to exogenous TH that does not accelerate the formation of adult structures such as limbs (26). However, their thyroid and pituitary glands develop early so that the role of TH in development of these frogs has not been established (27). The axolotl is considered to be an example of direct development, yet there are biochemical changes similar to ones that take place during anuran metamorphosis that occur spontaneously long before sexual maturity such as the appearance of new hemoglobins (28–30), serum albumin (29), and changes in muscle proteins (31). These cryptic transformations (32) can be induced prematurely by exogenously added TH suggesting that low levels of TH play a role in development long before sexual maturity just as is known to be the case for anurans and fish. No comparable studies have been reported for "obligatory neotenes" such as the mud puppy leaving open the possibility that although these animals are resistant to TH as adults, they too could have a TH-sensitive period much earlier during larval development.

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