

# Androgen-Induced Proliferation in the Developing Larynx of *Xenopus laevis* Is Regulated by Thyroid Hormone

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Exposure to exogenous androgen regulates cell number in the developing larynx of *Xenopus laevis* and hormone-regulated laryngeal development requires secretion of thyroid hormone (TH). We sought to determine whether exposure to TH is both sufficient and necessary for androgen-evoked cell proliferation (androgen competency) in developing larynx. Androgen competency was not observed in the premetamorphic larynx (tadpole stage 53, before TH secretion) but was present just prior to metamorphic climax (stage 58, during TH secretion). However, when TH is administered precociously (between stages 48 and 50), androgen competency can be observed at stage 53. The stage 52 larynx expresses high levels of the mRNA for TH receptor  $\alpha$ . The duration of TH exposure required at tadpole stage 48 is greater than 2 days; studies in juveniles indicate that TH exposure need not be maintained in order for androgen competency to persist. The effects of exposure to TH on androgen competency are long lasting and perhaps permanent. While organotypic cultures obtained from tadpoles during premetamorphosis (stage 52) can proliferate *in vitro* and proliferation is augmented by TH exposure as it is *in vivo*, precocious exposure to TH does not induce androgen competency. In contrast, androgen does evoke cell proliferation in cultures obtained from metamorphosing (stage 58) tadpoles; proliferation is confined to the cartilage component. Thus, unlike larynges *in vivo*, muscle will not proliferate in response to androgen, indicating the necessity for an additional factor not present *in vitro*. Androgen receptor mRNA expression, believed required for androgen competency, was assessed *in vivo* and *in vitro*. The tadpole larynx strongly expresses AR mRNA; expression does not require exposure to TH nor is expression diminished in culture. © 1996 Academic Press, Inc.

## INTRODUCTION

Differentiation of masculine and feminine phenotypes in vertebrates is directed by steroid hormones, particularly androgens and estrogens. Tissue responsiveness to steroids generally requires expression of functional receptors and is specific to cell type and developmental stage. The developmental factors that control the opening and closing of sensitive periods for steroid hormone action are largely unknown. In the sexually differentiated larynx of *Xenopus laevis*, muscle and cartilage cell numbers and types are controlled by androgen secretion during early postmetamorphic (PM) stages (reviewed in Kelley, 1996). The closing of the developmental sensitive period for cell addition in the lar-

ynx seems due to differentiation of androgen sensitive stem cells (Marin *et al.*, 1990). The opening of the sensitive period for androgen-evoked cell proliferation is the subject of this investigation.

The mature male larynx contains more muscle fibers and a larger and more complex cartilage than that of the female. Laryngeal anlage are present at tadpole stage 41; muscle begins to differentiate at tadpole stage 41 and cartilage at stage 43 (Nieuwkoop and Faber, 1956). The gonads sexually differentiate at tadpole stage 56 (Merchant-Larios and Villapando, 1981; Iwasawa and Yamaguchi, 1984; Robertson and Kelley, 1996) and by stage 62 masculinization of laryngeal innervation is apparent, though muscle fiber numbers are the same in the sexes (Kelley and Dennison, 1990; Robertson *et al.*, 1994). Muscle fiber number in males exceeds that of females by PM stage 1 (~3 months PM; Marin *et al.*, 1990); from PM1 on, muscle fiber type in the larynx diverges in the sexes (Tobias *et al.*, 1991a; Catz *et al.*, 1992). Sexual differentiation of laryngeal cartilages is first apparent

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at PM2 (~6 months PM; Sassoon and Kelley, 1986; Fischer *et al.*, 1995). During postmetamorphic stages, all laryngeal tissue types express androgen receptor (AR) and androgen receptor mRNA (Kelley *et al.*, 1989; Fischer *et al.*, 1995).

The onset of androgen-induced laryngeal masculinization coincides with the metamorphosis of the tadpole to the juvenile frog, a developmental epoch controlled by thyroid hormone (TH). Thyroxine ( $T_4$ ), which is secreted by the thyroid, is converted to the biologically active form triiodothyronine ( $T_3$ ) within responsive cells. If tadpoles are developmentally arrested before the beginning of metamorphosis using the  $T_4$  synthesis inhibitor propylthiouracil (PTU), androgen-induced laryngeal growth is specifically prevented (Robertson and Kelley, 1996). This observation raises the possibility that the sensitive period for androgen-induced changes in laryngeal cell number is controlled by TH secretion.

We wished to determine whether exposure to TH is necessary and sufficient for androgen-evoked cell proliferation in the larynx. The receptors for TH are expressed very early in development (Kawahara *et al.*, 1991) but secretion of TH is restricted to specific stages, beginning at tadpole stage 54, peaking at 60–62, and falling off by the end of metamorphosis at tadpole stage 66 (reviewed in Galton, 1983; Dodd and Dodd, 1976). Sex differences in laryngeal cell numbers are not apparent until juvenile stage PM1, 3–4 months after TH secretion is initiated. However, exposure to exogenous androgen can prematurely masculinize cell number and cell type in juveniles of both sexes (Sassoon *et al.*, 1986; Marin *et al.*, 1990; Tobias *et al.*, 1991b; Fischer *et al.*, 1995; Catz *et al.*, 1995). In addition, the developing larynx expresses AR mRNA from very early tadpole stages (Cohen and Kelley, 1994). We thus employed the induction of cell proliferation by exogenous androgen (androgen competency) as an assay for TH-induced androgen sensitivity before the onset of TH secretion, both in tadpoles and in parallel laryngeal organ cultures.

## MATERIALS AND METHODS

**Animals.** *X. laevis* tadpoles were obtained commercially (Nasco, Fort Atkinson, WI) or from mated pairs injected with 1000 units of human chorionic gonadotropin (Sigma). Embryonic and larval development of *X. laevis* has been divided into 66 stages based on morphological characteristics (Nieuwkoop and Faber, 1956). Stage 1 represents fertilization of the egg; embryonic development then proceeds to hatching at stages 35/36 (~50 hr postfertilization or PF). Larval or tadpole development (stages 37 through 66) has been further classified according to metamorphic status of the tadpole (Dent, 1968). The period from stage 37 to stage 53 (~24 days PF) is termed premetamorphosis; stage 54 marks the start of prometamorphosis or TH-directed metamorphosis. Forelimbs emerge at stage 58 (~44 days PF), and metamorphic climax corresponds to peak plasma TH levels and occurs at stages 59–62. Circulating TH then decreases and metamorphosis from tadpole to froglet is complete at stage 66 (~58 days PF).

Tadpoles were maintained at room temperature (~22–24°C) in Novaqua (Kordon, Hayward, CA)-treated filtered tap water in 2.5-liter polycarbonate tanks (25 tadpoles/tank) under a 12:12 L:D cycle and were fed nettle powder daily. In this population, the gonads of males and females do not become morphologically distinguishable until stage 56 (see Robertson and Kelley, 1996); sex cannot be determined at earlier stages. Postmetamorphic frogs were staged as described previously (Tobias *et al.*, 1991a).

**In vivo studies—Tadpoles.** Tadpoles were treated with dihydrotestosterone ( $5\alpha$ -androstan- $17\beta$ -ol-3-one; DHT, Sigma) and/or  $T_4$  (Sigma) as these are the most fully developmentally characterized, circulating forms of androgens and TH, respectively (Kang *et al.*, 1995; Leloup and Buscaglia, 1977). DHT was dissolved in 95% ethanol which was added to tank water to produce a final concentration of  $1.0 \times 10^{-7}$  M (the ethanol concentration was 0.0016%).  $T_4$  was solubilized in 0.5 M NaOH, added to tank water, and used at a final concentration of  $1 \times 10^{-8}$  M (100  $\mu$ M NaOH). At stage 45, tadpoles were randomly assigned to one of four experimental groups: untreated, DHT (stage 51–53),  $T_4$  (stage 48–50) followed by DHT (stage 51–53), or  $T_4$  (stage 48–50). The treatment paradigms are summarized in Fig. 1. In pilot studies, tadpoles were pretreated with  $T_4$  for 1, 2, or 7 days, beginning at stage 48. At stage 53, DHT-induced cell proliferation was observed only in the 7-day  $T_4$  treatment group; thus, this interval was chosen for subsequent studies. Four additional groups were also prepared: in one, endogenous TH synthesis was prevented by maintaining tadpoles from stage 48 (prior to the onset of TH secretion; Leloup and Buscaglia, 1977) in 0.01% 6-*n*-propyl-2-thiouracil (PTU, Sigma; this dose prevents metamorphic-associated laryngeal differentiation in *X. laevis*, Robertson and Kelley, 1996). In another, tadpoles were treated with  $T_4$  from stage 51 to 53. Two groups of stage 58 tadpoles were prepared. One was an untreated control group, and the other was treated with DHT for 3 days.

Tadpoles in each group received an injection of 10–20  $\mu$ Ci [ $^3$ H]-thymidine in 0.01% fast green into the peritoneal cavity using a Butterfly Infusion Set (Abbot Hospitals). After 30 min, animals were anesthetized by immersion in 0.1% MS222 (ethyl *m*-aminobenzoate methanesulfonic acid; Aldrich, Milwaukee, WI) and fixed overnight in 10% neutral buffered Formalin. They were then dehydrated through an ethanol series, cleared with methyl salicylate, and embedded in Paraplast.

**In vivo studies—Juveniles.** To determine whether continued TH exposure is required for androgen-evoked cell proliferation, PM animals were studied. PM2 animals (~6 months PM) were chosen for study as the larynx evinces a robust proliferative response to androgen at this stage (Sassoon *et al.*, 1986; Fischer *et al.*, 1995). TH synthesis was blocked by maintaining PM2 frogs in 0.01% PTU for 3 weeks. PTU-treated and control frogs were implanted with DHT-filled (0.25 mg/g body weight) or empty silastic tubing in the dorsal lymph sac for 5 days; they then received a [ $^3$ H]thymidine injection (1  $\mu$ Ci/g body weight), were killed 1 h later (see Fischer *et al.*, 1995 for details), and tissue was processed for autoradiography as described above.

**In vitro studies.** Larynges were removed from stage 52 or stage 58 tadpoles and cultured in Opti-MEM I Reduced-Serum media (Gibco) supplemented with gentamycin (50  $\mu$ g/ml), penicillin (50 IU/ml), streptomycin (50 IU/ml), fungizone (0.125  $\mu$ g/ml), and stripped fetal bovine serum (4%). Fetal serum was stripped of steroid and thyroid hormones (see Ishizuya-Oka and Shimozawa, 1991) using dextran-coated charcoal. Norit-A (1.25 g), Dextran T-70 (0.125 g), and 12.5 ml FBS were stirred for 2 hr at 4°C, an addi-

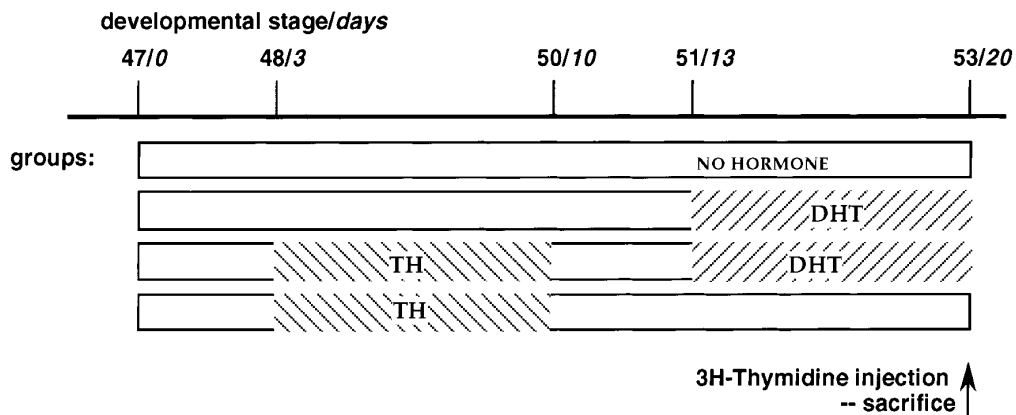


FIG. 1. Diagrammatic representation of the experimental paradigm illustrating the time course of administration of thyroxine ( $T_4$ ) and/or dihydrotestosterone (DHT). All tadpoles received an injection of [ $^3H$ ]thymidine at stage 53 and were killed 30 min later.

tional 100 ml serum was then added, and the mixture stirred overnight and then centrifuged for 30 min at 10,000g, after which the stripped serum was added to the medium. Because  $T_4$  might not be as efficiently converted to the more active form triiodothyronine ( $T_3$ ) *in vitro* as in tadpoles, for laryngeal explants  $T_3$  (Sigma) was used.  $T_3$  was dissolved in 0.5 M NaOH and added to the medium to produce a final concentration of  $1 \times 10^{-8}M$ .

Hormone treatment paradigms paralleled those used *in vivo*; proliferating cells in all explants were labeled at Day 16. Culture medium was supplemented with [ $^3H$ ]thymidine (4  $\mu Ci/ml$ ) for 4 hr. The laryngeal explant was then fixed in 10% neutral buffered Formalin for at least 24 hr. Tissue was then cresyl violet stained *en bloc* and embedded in paraplast as described above.

The development of laryngeal explants was also followed with the aid of bromodeoxyuridine (BrdU, Boeringer-Mannheim) immunocytochemistry in order to visualize zones of proliferation in the entire explant in whole mount. The culture medium was supplemented with 50  $\mu M$  BrdU at Day 16 for 4 hr. Explants were rinsed with culture medium and fixed with ice-cold absolute ethanol. Tissue was then hydrated in ethanol and incubated in 2 N HCl with 0.5% Tween 20 at 37°C for 8 min. A monoclonal antibody against BrdU (Boeringer-Mannheim) was used at a dilution of 1:100 overnight at 4°C. Tissue was incubated for 1 hr with Texas red-conjugated goat anti-mouse (Jackson ImmunoResearch; preadsorbed with laryngeal cartilage at room temperature for 1 hr) at room temperature. After rinsing with PBS, the tissue was mounted in glycerol and then examined using confocal laser microscopy.

**Expression of androgen and thyroid hormone receptor mRNA in larynx.** To determine whether larynx expresses the relevant receptors, expression of AR mRNA (He *et al.*, 1990) and TH receptor  $\alpha$  ( $TR\alpha$ ) mRNA (Yaoita *et al.*, 1990) was examined using *in situ* hybridization as in Fischer *et al.* (1995) with the following modifications. The AR mRNA probe was the cDNA fragment described previously (He *et al.*, 1990) which contains a portion of the ligand-binding and DNA-binding domains. The  $TR\alpha$  probe used was transcribed from pXTR $\alpha$ AA/BS (gift of D. Brown), a cDNA from the constitutively expressed  $TR\alpha$  mRNA (Yaoita *et al.*, 1990). The  $TR\alpha$  probe was used only on tadpole stage 52 larynges. Tadpole larynges were collected and fixed overnight in

4% paraformaldehyde at 4°C. Cultured laryngeal explants were fixed in 4% paraformaldehyde for 2 hr at room temperature followed by immersion in cresyl violet solution. Tissue was then dehydrated through an ethanol series and cleared in methyl sali-

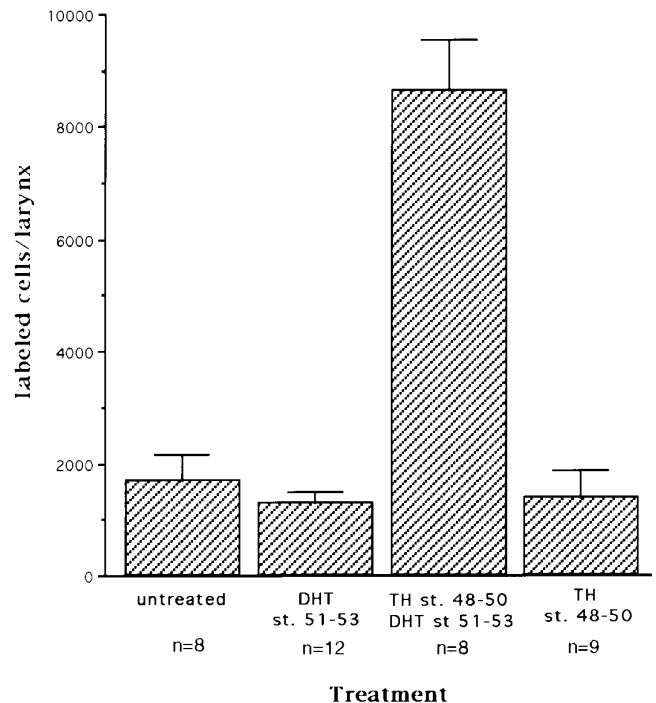


FIG. 2. The effects of hormone treatment on number of labeled cells per larynx in tadpoles; treatment groups as in Fig. 1. Values given are means  $\pm$  standard error of the mean (SEM); the number of animals in each group is indicated. The mean value for the TH/DHT group differed significantly from the mean value of the untreated group; the mean values for the other groups did not differ significantly from the control value.

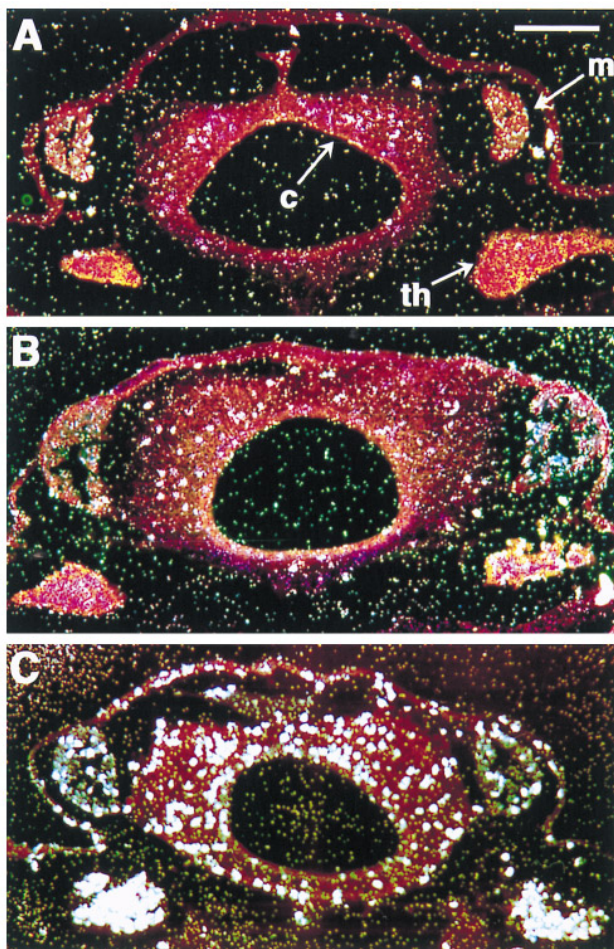


FIG. 3. Representative transverse autoradiographic sections through the larynx of stage 53 tadpoles prepared following [ $^3\text{H}$ ]-thymidine injection. Sections were exposed to nuclear emulsion for 6 weeks, developed, and counterstained with cresyl violet. (A) Untreated. (B) DHT-treated from stage 51–53. (C) TH-treated from stage 48–50 and then DHT-treated from stage 51–53. Scale bar, 100  $\mu\text{m}$ . Abbreviations: m, muscle; c, cartilage; th, thyohydrals.

cylate before being embedded in Paraplast. Ten-micrometer sections were affixed to Superfrost-Plus (Fisher) microscope slides by overnight drying at 40–50°C and then stored at 4°C until hybridization. Sections were deparaffinized in xylene, refixed in paraformaldehyde, and treated with Proteinase K (3  $\mu\text{g}/\text{ml}$ ) for 20 min. The tissue was then incubated in 0.2 N HCl followed by acetic anhydride. Slides were incubated for 16 hr in a humid chamber at 50°C with  $2 \times 10^6$  cpm of probe, washed in  $2 \times$  SSPE, treated with RNase A (20  $\mu\text{g}/\text{ml}$ ), and washed again in 50% formamide/ $2 \times$  SSPE at 55°C. The slides were then dipped in NTB-2 and exposed for 3–5 weeks. After developing in Kodak D-19, sections were counterstained with cresyl violet.

**Autoradiography and data analysis.** Tissue from both *in vitro* and *in vivo* experiments was serially sectioned at 10  $\mu\text{m}$ . After clearing in xylene and rehydrating through an ethanol series, slides were dipped in nuclear track emulsion (NTB-2, Kodak) and exposed for 6 weeks (*in vitro*) or 3 weeks (*in vivo*) at 4°C. Slides were developed in Kodak D-19 developer and counterstained with cresyl violet.

For each tadpole, the total number of labeled cells per larynx was determined by counting labeled cells in every sixth section and then multiplying by 6. A group mean and standard error of the mean was then calculated for each treatment group. A labeling index was also determined as described previously (Fischer *et al.*, 1995). Counts of labeled cells were determined for three fields (170  $\mu\text{m}^2$ ) per larynx from an area adjacent to the thyohydrals. The ratio of the number of labeled nuclei divided by the total (labeled plus unlabeled) number of nuclei was determined for each field and averaged to yield a labeling index for each larynx. For laryngeal cultures from stage 53 tadpoles, three, equally spaced, transverse sections through (25, 50, and 75%) the larynx were chosen, the number of labeled cells in the entire section was counted, an average per section obtained, and a total determined by multiplying by the total number of sections. For laryngeal cultures from stage 58 tadpoles, six equally spaced sections were used. Because orientation of the sections was difficult to determine in explants, the above method provided a more representative estimate of proliferation than that used for *in vivo* studies.

**Statistical analysis.** Differences in the number of labeled cells in stage 53 tadpole larynges examined *in vivo* and *in vitro* were assessed using a one-way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) as a post-hoc test (two-tailed distribution,  $P \leq 0.05$ ; Statview, Abacus, Berkeley, CA). For the stage 58 larynges, a Student's *t* test (two-tailed distribution,  $P \leq 0.05$ ) was used to compare the number of labeled cells with and without DHT treatment, for each tissue type (muscle or cartilage) under the two observation conditions (*in vivo* and *in vitro*). To assess differences in labeling indi-

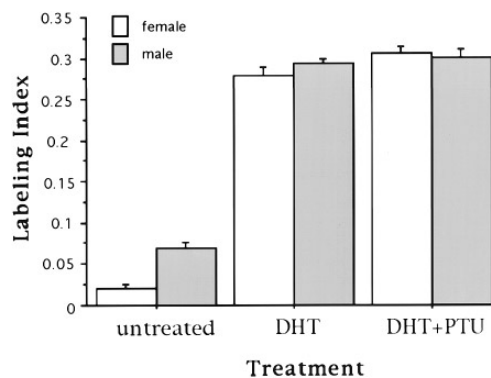


FIG. 4. The effects of hormone treatments on laryngeal cell proliferation in PM2 juveniles. The number of labeled cells/total number of cells (labeling index) was determined for each larynx; values given are means  $\pm$  SDs and the number of animals was 3 for each group. The labeling index was significantly higher in males than in females but treatment with DHT significantly increased the labeling index in both sexes. Blockade of TH synthesis with PTU did not affect DHT-induced cell proliferation.

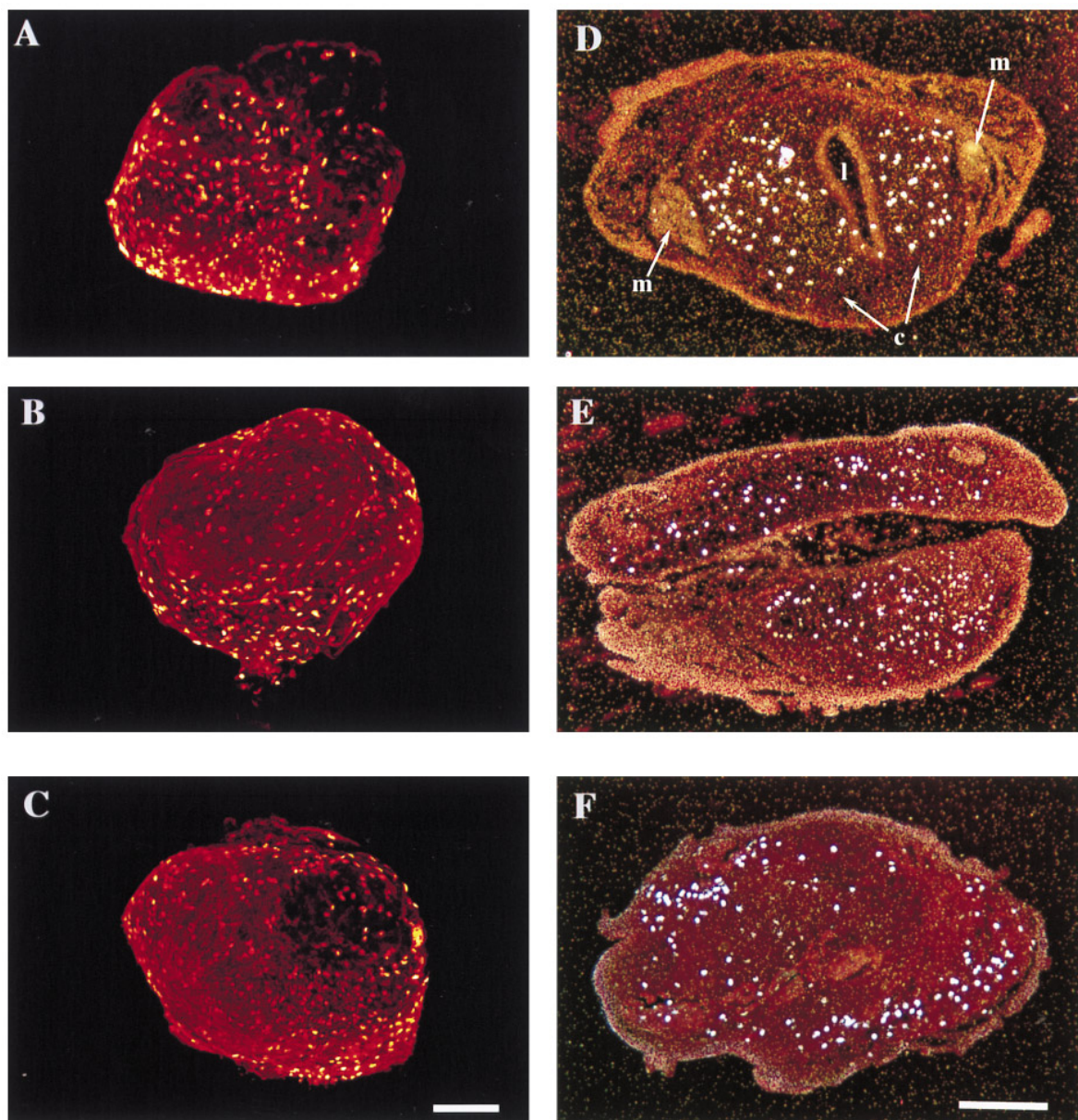


FIG. 5. Cell proliferation demonstrated in laryngeal explants from stage 52 tadpoles examined following administration of BrdU in whole mounts (A–C) or following administration of [ $^3$ H]thymidine in 10- $\mu$ m sections (D–F). Explants were untreated (A, D), DHT-treated (B, E), or TH/DHT-treated (C, F). Bar, 100  $\mu$ m. Abbreviations: m, muscle; c, cartilage; l, lumen.

ces, the Mann–Whitney  $U$  test was used (two-tailed distribution,  $P \leq 0.05$ ; Statview, Abacus).

## RESULTS AND SPECIFIC DISCUSSION

*Androgen-induced cell proliferation in the tadpole larynx requires exposure to thyroid hormone.* Exposure to

exogenous androgen can evoke cell proliferation in the tadpole larynx if preceded by treatment with TH (Fig. 2). Responses to hormone treatments were evaluated by determining the total number of labeled cells per larynx; group means were evaluated using an ANOVA which revealed a significant main effect of treatment ( $P < 0.0001$ , PLSD). Post-hoc analyses revealed no significant difference between untreated animals and those exposed to DHT be-

tween stages 51 and 53 ( $P = 0.5$ ). However, pretreatment with  $T_4$  between stages 48 and 50 does lead to DHT-induced cell proliferation. The number of labeled cells in tadpole larynges pretreated with  $T_4$  from 48 to 50 and then given DHT from 51 to 53 differed significantly from that observed following DHT alone ( $P < 0.0001$ ). The enhanced number of labeled cells seen after  $T_4$ /DHT treatment was not simply the effect of the  $T_4$  treatment from 48 to 50; there was no significant difference in total number of labeled cells/larynx in the  $T_4$  from stage 48 to 50 group compared to that of untreated controls ( $P = 0.1$ ). In order to determine whether the ongoing proliferation at stage 53 was affected by treatment-induced proliferation at earlier stages (which could have affected stem cell availability or total cell numbers) and to compare results across experiments, we also obtained a labeling index for each larynx. Results were similar to those for total number of labeled cells per larynx: the labeling index for the  $T_4$ /DHT treatment group was  $17.9 \pm 1.8\%$  vs  $6.0 \pm 0.8\%$  for the untreated or  $3.9 \pm 0.4\%$  for the DHT-only-treated groups ( $P < 0.0001$  for both comparisons; the labeling index for the DHT-only group does not differ significantly from that of the untreated control;  $P = 0.3$ ).

*In vivo TH/DHT treatment induces proliferation specifically in both muscle and cartilage of the larynx.* At stage 53, the tadpole larynx is made up of differentiating cartilage and muscle; the hyaline cartilages are beginning to form and are flanked bilaterally by muscle masses which have not yet assumed their bipinnate, adult form. A brief pulse of [ $^3$ H]thymidine reveals ongoing cell proliferation in both cartilage and muscle of the larynx (Fig. 3A). Proliferating cells are most prevalent in developing cartilage. The extent and pattern of cell proliferation in tadpoles treated with DHT from stage 51 to 53 (Fig. 3B) does not differ from untreated controls (Fig. 3A). However, following pretreatment with TH, DHT evokes a marked enhancement of cell proliferation throughout developing cartilages and muscle, including the associated thyohyal cartilages (Fig. 3C).

Thus, precocious exposure to TH induces the ability to respond to exogenous androgen treatment with cell proliferation (androgen competence). Of the tissues we have examined, androgen competence is thus far confined to laryngeal muscle and cartilage. Competence is not induced in other tadpole tissues which do not normally respond to androgen, such as the pharynx (data not shown).

*TH is not required for ongoing laryngeal cell proliferation in premetamorphic tadpoles or for DHT-evoked cell proliferation in juveniles.* We next wished to determine whether there is any endogenous TH secretion prior to stage 53, secretion that might be responsible for the ongoing cell proliferation observed in control larynges (Fig. 3A) but insufficient for androgen competency (Fig. 3C). A group of tadpoles was treated with PTU from stage 48 to block all TH synthesis. When examined at stage 53, the number of proliferating cells/larynx (mean: 1645; SEM:  $\pm 70$ ;  $N = 3$ ) did not differ from that of untreated controls ( $1703 \pm 467$  labeled cells,  $N = 8$ ,  $P = 0.9$ ). Thus, ongoing cell proliferation in

the larynx prior to stage 53 appears independent of endogenous TH secretion.

Continued exposure to TH is not required for DHT-evoked cell proliferation (Fig. 4). In male and female juveniles (PM2), DHT treatment for 5 days produces a significant difference in labeling index compared to untreated controls. A Mann-Whitney  $U$  test indicates a significant effect of both sex ( $P < 0.05$ ; untreated males vs untreated females) and treatment ( $P < 0.05$ ; untreated vs DHT-treated, both males and females). The DHT-induced increase in labeling index was also observed in the presence of PTU, a TH synthesis inhibitor; values did not differ significantly from animals treated with DHT alone (males:  $P > 0.5$ ; females:  $P > 0.1$ ).

*Requirements for androgen competency in cultured larynges.* We next wished to determine whether androgen and TH act directly on laryngeal tissue. Larynges were removed prior to TH secretion (stage 52) and explant development was examined. BrdU or [ $^3$ H]thymidine was added to the medium to assess the viability and morphology of the cultures, as well as the extent of cell proliferation (Figs. 5A and 5D). As is the case *in vivo*, proliferating cells are found throughout the tissue (Fig. 5A) and are more prominent in cartilage than in muscle (Fig. 5D). The orientation of the cultures is more difficult to control during sectioning than the orientation of tadpole larynges. Nonetheless, a favorable plane of section (Fig. 5D) reveals good preservation of laryngeal morphology for as long as 16 days in culture (compare Fig. 5D with Fig. 3A).

The effects of hormone treatment *in vitro* were assessed both qualitatively using BrdU and quantitatively using [ $^3$ H]thymidine. In neither case did DHT, with or without prior TH treatment, induce cell proliferation over values present in control tissue (Figs. 5B and 5E; Figs. 5C and 5F). ANOVA of labeled cells per larynx in each group (Fig. 6) revealed no significant contribution of hormone treatment ( $P = 0.3$ ).

Thus, while administering TH to tadpoles induces androgen competency in the premetamorphic larynx, administering TH to isolated premetamorphic larynges in culture does not. One possible explanation for this failure of response is loss of sensitivity to hormones either via a failure of hormone receptor expression or via a failure of hormone-evoked proliferative response. Since TH itself can be a mitogen, we tested its effects *in vitro* and compared results to a similar *in vivo* group. First we determined whether the TR is expressed in the tadpole larynx using an antisense probe to the TR $\alpha$  mRNA; high levels of expression are present at tadpole stage 52 in larynx (Fig. 7C). When administered to tadpoles,  $T_4$  exposure from stages 51 to 53 significantly increased the number of [ $^3$ H]thymidine-labeled cells compared to controls ( $3548 \pm 1016$ ;  $n = 5$  vs  $1703 \pm 467$ ,  $n = 8$ ;  $P = 0.01$ ). A similar effect is observed *in vitro* when  $T_3$  is given from Days 10 to 16 ( $5579 \pm 1155$ ;  $n = 18$  vs  $2698 \pm 793$ ,  $n = 17$ ;  $P = 0.01$ ). Thus, laryngeal explants can respond to TH with cell proliferation and the response resembles that seen in tadpoles in which the larynx expresses a TR gene.

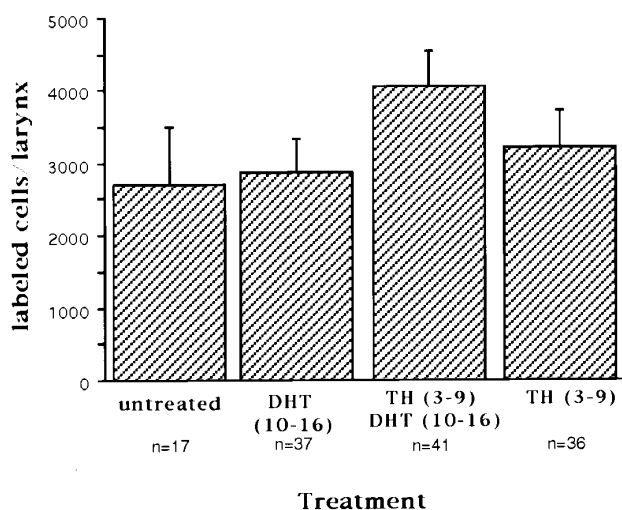


FIG. 6. The effect of hormone treatment on number of labeled cells per larynx in explants; treatment groups as in Fig. 1. Values given are means  $\pm$  SEM; the number of explants in each group is indicated. There was no significant difference in the total number of [ $^3$ H]thymidine-labeled cells between untreated controls and any hormone treatment group.

Alternatively, the difference between *in vitro* and *in vivo* results could be the result of loss of AR in culture. Studies *in vitro* indicate that high levels of AR mRNA expression are present after 16 days in culture (Fig. 7D). No effect of DHT treatment with (Fig. 7E) or without (not shown) preexposure to TH is apparent. No hybridization signal is apparent in larynx when sense AR probes are applied (Fig. 7F). High levels of AR mRNA in larynx can also be detected *in vivo* at stage 48 (Fig. 7A), the earliest stage used in this study. When PTU treatment is begun at stage 48, and AR mRNA expression examined at stage 54, high levels of expression are again apparent (Fig. 7B). We conclude that exposure to TH is not required to maintain AR mRNA expression and that induction of androgen competency is not regulated at the level of AR mRNA transcription.

Finally, we explored the possibility that a TH-induced, extralaryngeal influence is required for androgen competency by examining responses of explanted stage 58 larynges. By stage 58, endogenous TH secretion has been ongoing for approximately 18 days. As in stage 53 tadpoles, a pulse of [ $^3$ H]thymidine reveals ongoing proliferation in both laryngeal muscle and cartilage (Fig. 8). DHT increases the number of proliferating cells within the cartilage, both *in vivo* ( $P < 0.005$ ) and *in vitro* ( $P < 0.05$ ; Fig. 8A). However, while cells within laryngeal muscle also proliferate in response to DHT *in vivo* ( $P < 0.005$ ), cells within laryngeal muscle do not proliferate in response to DHT *in vitro* ( $P = 0.98$ ; Fig. 8B). We conclude that androgen competency is present in all tissue compartments of the tadpole larynx during metamorphosis (stage 58) but not before (stage 53). While andro-

gen-evoked proliferation can be demonstrated by both laryngeal cartilage and muscle in tadpoles, androgen competency can only be demonstrated in cartilage in isolated larynges, suggesting that an extralaryngeal influence may be required for muscle.

## DISCUSSION

The control of cell proliferation by steroid hormones plays an important role in the development of target tissues. The ability of these hormones, primarily androgens and estrogens, to induce mitotic activity is often developmentally regulated and can be confined to precise sensitive periods (reviewed in Kelley, 1986; 1992). The factors which govern the opening and closing of these periods are by and large unknown. The larynx of *X. laevis* is sexually dimorphic with respect to cell number as a result of an androgen-driven program of cell proliferation (Sassoon *et al.*, 1986; Sassoon and Kelley, 1986; Marin *et al.*, 1990; Watson *et al.*, 1993). We show here that the opening of the sensitive period for androgen-evoked cell proliferation in the larynx (androgen competency) is due to exposure to another hormone, thyroxine. Our results indicate that early exposure to TH permits precocious androgen-evoked cell proliferation. The time course of androgen competency in untreated animals reflects secretion of endogenous TH; androgen can induce cell proliferation at stage 58, but not at stage 53. If TH secretion is blocked, androgen cannot evoke laryngeal growth (Robertson and Kelley, 1996). The effects of exposure to TH on androgen competency are long lasting and perhaps permanent. We thus conclude that, in the tadpole, exposure to TH is both necessary and sufficient for androgen competency.

Thyroid hormone is a very powerful developmental agent in amphibia. During metamorphosis, tissues that express the TR remodel in a dramatic fashion, the tail, for example, regresses entirely while the limbs erupt. TH-induced changes differ in threshold; some require prolonged exposure to high levels of hormone while others are more readily evoked (Brown *et al.*, 1995). Our studies indicate that, at stage 48, a week of TH treatment is sufficient to induce androgen competency, whereas 1 or 2 days is not. During the week of precocious TH treatment, the tadpole advances from stage 48 to stage 50. This time course is consistent with results obtained by Brown and colleagues who have explored TH-induced programs of gene regulation in the tail and limb during metamorphosis (Buckbinder and Brown, 1992; Shi and Brown, 1993; Brown *et al.*, 1996); a conclusion of these studies is that a TH exposure of at least 2 days is necessary to initiate the complex developmental programs required during metamorphosis. The gene program required for TH-induced androgen competency is likely to be similarly complex.

The widespread effects of TH are reflected in the almost ubiquitous expression of its receptors (Kawahara *et al.*, 1991). Our results indicate that developing laryngeal carti-

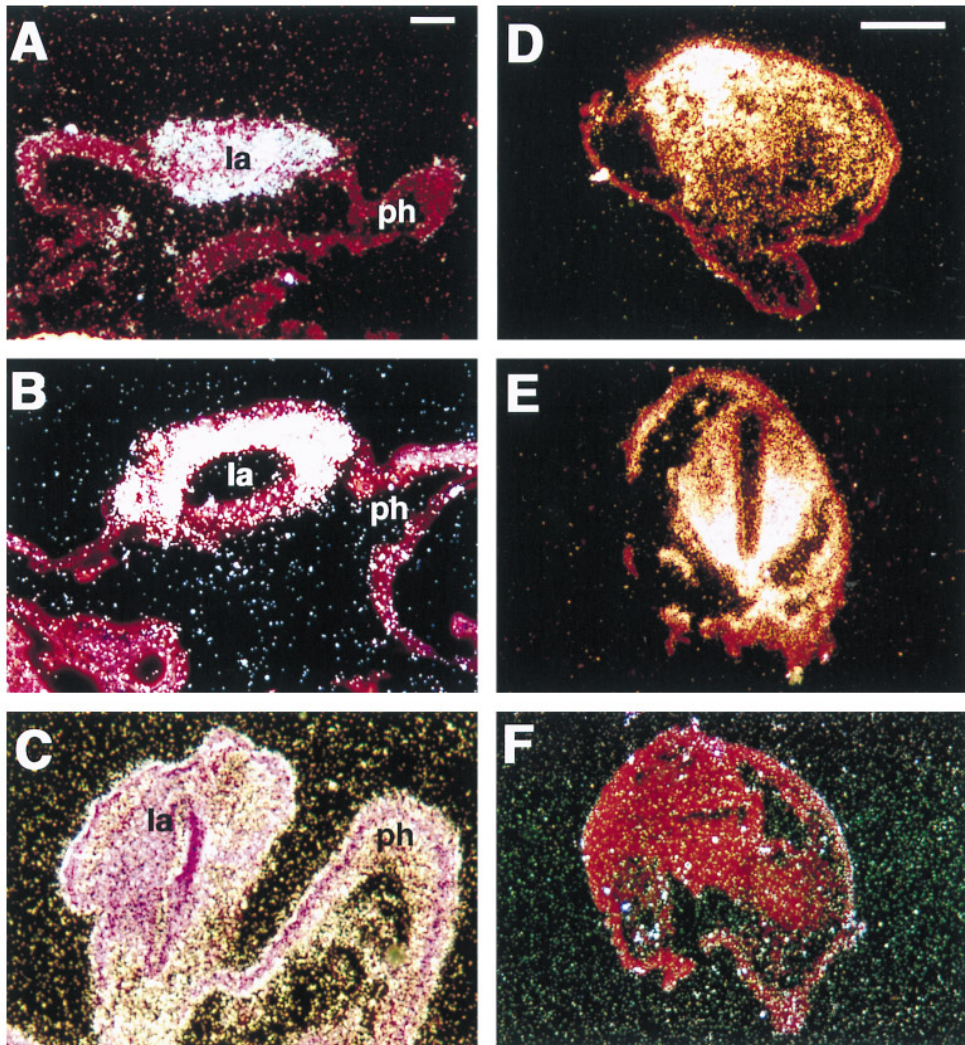


FIG. 7. Hormone receptor gene expression in tadpole (A–C) and explant (D–F) larynges. Sections were hybridized *in situ* to  $S^{35}$  riboprobes from the androgen receptor (A, B, D, E) or a sense control (F) or to the  $\alpha$  isoform of the TR (C). AR mRNA is expressed in larynx, but not pharynx, as early as tadpole stage 48 (A) and expression is also present at stage 54, even in the presence of PTU (B). In laryngeal explants, AR mRNA expression is sustained after 16 days, with no apparent difference between untreated control explants (D) and TH/DHT-treated explants (E). At stage 52, TR $\alpha$  mRNA is expressed in both larynx and pharynx (C).

larynges and muscle express TR $\alpha$  mRNA as early as stage 52. Because these receptors are nuclear transcription factors, the molecular mechanisms through which TH exerts its effects can be illuminated by examining the TH-regulated gene networks. A clear result obtained by Brown and his colleagues is that TH alters the expression of a different set of genes depending upon the target tissue. The maturation of metamorphic tail (resorption) and limb (growth) are very different processes regulated by the same hormone, TH. We might well expect, therefore, that there are specific genes regulated by TH in the larynx, and possibly other androgen-sensitive tissues, that participate in the unique develop-

mental cascade leading to masculinization. The present results allow us to exclude the AR as a gene transcriptionally regulated by TH; AR is expressed before TH secretion and is maintained even in the absence of any TH (produced by PTU-induced blockade of T<sub>4</sub> synthesis).

Androgen competency does not require that TH be present simultaneously with DHT. In the present experiments, TH and DHT exposures were separated by 3 days. More than 2 days of TH treatment was required for the induction of androgen competency. In addition, DHT evokes cell proliferation in postmetamorphic frogs (Sassoon *et al.*, 1986) and blockade of T<sub>4</sub> synthesis with PTU had no effect on

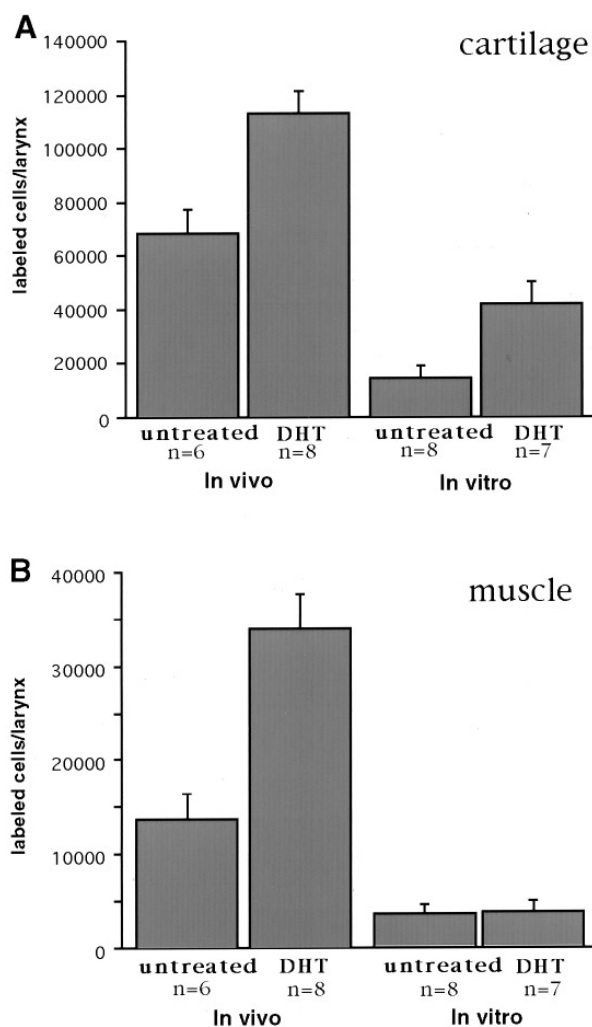


FIG. 8. The effect of DHT treatment on cell proliferation of stage 58 larynges. Values given are means  $\pm$  standard error of the mean (SEM); the number of animals in each group is indicated. In cartilage (A), DHT induced a significant increase in the number of labeled cells both *in vivo* and *in vitro*. In muscle (B), DHT only induced a significant increase in labeled cells *in vivo*.

DHT-induced proliferation (present study). We can conclude that a relatively brief period of exposure to TH results in a long-lasting change in the ability of androgen to induce laryngeal cell proliferation. This change can be induced as early as tadpole stage 53; during normal development, the effects of androgen are not manifest until postmetamorphic stages when androgen is required for male-specific muscle fiber addition (Sassoon and Kelley, 1986; Marin *et al.*, 1990).

Androgen competency is, however, present by stage 58. At this stage, both cartilage and muscle proliferate in response to DHT *in vivo*. In culture, only cartilage exhibits DHT-induced proliferation, indicating that muscle requires

an additional factor. One possible requirement for the proliferation of myoblasts is innervation. At PM2, DHT-induced laryngeal muscle fiber addition is only observed in the innervated side of a unilaterally denervated female larynx (Tobias *et al.*, 1993). These data suggest that the nerve may be supplying myoblasts with a trophic factor necessary for proliferation; this factor may or may not itself require androgen for its production (i.e., androgen may act on laryngeal motor neurons). This relation between two, mutually dependent, androgen-sensitive cell types could be a mechanism for refining a proliferative response which would otherwise continue unchecked.

The influence of TH on endocrine sensitivity has been explored in another endocrine-regulated system in *X. laevis*, estrogen regulation of vitellogenesis (reviewed in Wahli *et al.*, 1981; Rabello *et al.*, 1994). The ability of estrogen, which—like DHT—is a gonadal steroid, to activate the liver-specific vitellogenin gene (estrogen competency) is developmentally regulated. Like the androgen-mediated proliferation in the larynx, estrogen-induced vitellogenin synthesis is not a normal feature of tadpole development but instead provides a model system to study the determination of steroid sensitivity. The onset of estrogen competency coincides with metamorphosis and is confined to a fraction of hepatocytes (Kawahara *et al.*, 1987). In culture, exogenous TH induces hepatocyte precursors to become estrogen competent (Kawahara *et al.*, 1989). Estrogen competency can be induced by precocious administration of TH. A feature common to the induction, by TH, of both laryngeal androgen and liver estrogen competency is that the TH exposure produces a long-lasting, perhaps permanent, effect. In both cases, only a pulse of TH is required. High plasma titers of TH exist during a relatively brief period of development spanning the few weeks of the metamorphic climax. It is this period of secretion that is responsible for cellular changes which last through adulthood.

In many instances, TH-induced remodeling of tadpole tissues can be recreated *in vitro*. Isolated tail and limbs, for example, can be induced to regress or develop, respectively (Tata *et al.*, 1991), and the intestine will develop its adult epithelial form in culture (Ishizuya-Oka and Shimozawa, 1991). Biochemical changes also occur *in vitro*, as demonstrated by the estrogen competency with respect to vitellogenin synthesis described above (Kawahara *et al.*, 1989). However, we did not observe the same effects of TH on androgen competency in cultured larynges that we observed in tadpoles. TH exposure did not result in DHT-augmented laryngeal cell proliferation *in vitro*. The lack of response was not due to a failure of proliferative capacity in culture because both TH, in stage 52 cultures, and DHT, in stage 58 cultures, could evoke cell proliferation. Neither was this failure due to culture-association loss of AR expression; AR mRNA expression was maintained. Another explanation for the difference between *in vivo* and *in vitro* results is that some general maturation process of the larynx cannot take place in culture. Such a process might, for example, be oc-

curing between stages 48 (when we begin TH treatment) and 58 (when endogenous TH titers are high) and could be connected with developmental changes in innervation (see above) or in circulating factors.

In summary, we have analyzed the onset of androgen sensitivity in the larynx of *X. laevis* and the contribution of TH to this process. The larynx undergoes remodeling during development in response to androgen, resulting in a sexually dimorphic vocal system. The onset of sensitivity to androgen in the larynx, or androgen competency, is regulated by TH. We can conclude that a major action of TH is controlling the temporal boundaries for sensitive periods of steroid hormone action. An understanding of how these temporal boundaries are created and impact on embryogenesis now requires analysis of the molecular mechanisms involved.

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