

Prolactin Opens the Sensitive Period for Androgen Regulation of a Larynx-Specific Myosin Heavy Chain Gene

Christofer J. Edwards,¹ Kazutoshi Yamamoto,² Sakae Kikuyama,² Darcy B. Kelley¹

¹ Department of Biological Sciences, MC 2432, 911 Fairchild, Columbia University, New York, New York 10027, USA

² Department of Biology, School of Education, Waseda University, Nishiwaseda, Shinjuku, Tokyo, 169-8050, Japan

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ABSTRACT: The larynx of *Xenopus laevis* is a sexually differentiated vocal organ in which male muscle is entirely fast twitch and expresses high levels of a fast twitch myosin heavy chain gene, LM. Female muscle, however, is mostly slow twitch and expresses little LM. Androgen is unable to induce expression of LM until after metamorphosis is complete. The expression of LM during metamorphic and early postmetamorphic development parallels secretion and expression of the pituitary hormone prolactin. Here, we show that exposure to prolactin is necessary to allow androgen-induced LM expression in postmetamorphic froglets. In prolactin-

deprived animals, androgen-induced changes in the contractile properties of laryngeal muscle are blocked, which prevents the rapid rates of muscle contraction required for males to produce courtship songs. Thus, prolactin opens the sensitive period for androgen-induced LM expression in the larynx and controls the ability of male sex hormones to masculinize the vocal system both at the level of gene expression and vocal organ physiology.

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The influence of steroid hormones on sexual differentiation of target tissues is typically confined to a limited sensitive period during development. The factors which open and close hormonal sensitive periods are largely unexplored and the subject of our research. We have approached this question by examining androgen-directed development of the larynx, or vocal organ, of *Xenopus laevis*, a process responsible for the expression of sexually differentiated phenotypes in the adult (reviewed in Kelley, 1996). The adult male larynx contains entirely fast twitch muscle fibers, whereas that of the female contains predominantly slow twitch fibers (Sassoon et al., 1987). The exclusively fast twitch composition of male laryngeal mus-

cle allows him to make his characteristic fast (70-Hz) advertisement call. In the female, heterogeneous laryngeal muscle composition limits vocal production to slow (6-Hz) clicks (Tobias et al., 1991a).

The developmental program of the male larynx—which includes the transformation of muscle fiber types from slow to fast—begins to diverge from that of the female after metamorphosis. At the end of metamorphosis, juvenile larynges are predominantly slow twitch in fiber type in both sexes; males begin to convert slow twitch to fast twitch fibers about 6 months later and the larynx achieves its adult phenotype between 6 and 18 months later (between PM5 and PM6) (Tobias et al., 1991a). Castration halts but does not reverse muscle fiber type switching; fiber type switching can be reinstated in castrated adults with exogenous androgen and exogenous androgen treatment can precociously convert muscle fibers to

Correspondence to: D. B. Kelley

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fast twitch in juvenile males (Tobias et al., 1991b). Females given exogenous androgen or a testis transplant will convert slow fibers to fast at any developmental stage (Tobias et al., 1991b; Watson et al., 1993). Thus, while the larynx is permanently masculinized in fiber type by exposure to androgen, in its absence the larynx maintains its sensitivity to hormone.

What opens the sensitive period for androgen response? To approach this question, we identified an laryngeal-specific myosin heavy chain gene, LM, whose expression contributes to muscle fiber type transformation. Every muscle fiber in the adult male larynx is fast twitch and every fiber expresses LM; in females only a subset of fibers is fast twitch and expresses LM (Catz et al., 1992). At 6 months post-metamorphosis (PM2), LM expression is higher in male than in female larynges; however, castration at the end of metamorphosis blocks LM expression (Catz et al., 1995). When is LM first expressed and when does it come under the control of androgen? One possibility is that androgen alone regulates LM expression in the larynx. If this were the case, one would expect LM to be expressed in the tadpole larynx, since androgen is present and the androgen receptor gene is expressed throughout tadpole stages (Kang et al., 1995; Cohen and Kelley, 1996). Another possibility is that androgen-regulated LM expression is triggered by another hormone; androgen-induced cell proliferation is, for example, triggered by secretion of thyroid hormone (Robertson and Kelley, 1996; Cohen and Kelley, 1996).

To address these questions, we examined LM expression at different stages of development in males and females. Identification of a specific myosin heavy chain gene can be problematic because of the sequence similarity between different myosins. To circumvent this problem, we used ribonuclease protection assay (RPA), as it is sensitive to very small differences in sequence. Here, we report that LM expression is first detected during metamorphic climax but increases markedly only after metamorphosis is complete. Prolactin is greatly up-regulated at the end of metamorphosis in *Xenopus* and remains high throughout postmetamorphic development (Buckbinder and Brown 1993; Yamamoto et al., 1995). To determine whether LM expression is regulated by prolactin and whether androgen and prolactin effects are synergistic, we manipulated prolactin levels via exogenous administration and hypophysectomy in the presence and absence of androgen. Prolactin has a strong synergistic effect on androgen regulation of LM expression, an effect which contributes to androgen-regulated LM expression during early juvenile stages. In addition, depriving animals of prolactin

blocks androgen's effects on the contractile properties of the laryngeal muscle, which results in tetany at the high rate of contraction characteristic of mate calling.

MATERIALS AND METHODS

Animals and Surgical Procedures

Xenopus laevis tadpoles and juveniles were obtained from Nasco (Fort Atkinson, WI). Tadpoles were assigned to stages according to Nieuwkoop and Faber (1956) and approximate days postfertilization (pf) given here are taken from this Normal Table. Postmetamorphic (PM) animals were staged by body and larynx weight according to Tobias et al. (1991a); animals examined 1 month after metamorphosis is complete are referred to here as being at PM0. Gonadectomy and hypophysectomy were performed under anesthesia in MS-222 (immersion in a 0.16% solution of ethyl *m*-amino benzoate methane sulfonic acid; Sigma). The gonads and associated fat bodies were removed via an abdominal incision (as described in Marin et al., 1990). For sham gonadectomies, only fat bodies were removed. The pituitary was removed via an incision at the base of the skull using an angled micropoint hook. For sham hypophysectomies, the skull was incised with a scalpel (tadpoles) or opened with a dental drill (PM2).

RNA Extraction of Larynges

For each experimental group, larynges were pooled and homogenized (for total RNA extraction) using a Polytron homogenizer. RNA was isolated with RNazol (Tel-Test; extraction procedures as specified by the manufacturer). RNA was quantified at 260 nm using ultraviolet spectrophotometry. To check for possible degradation, 3 µg of RNA from each sample was run on an agarose gel.

Ribonuclease Protection Assay

We assayed LM expression using RPA. ³²P CTP (PB 20382, specific activity 800 Ci/mmol; Amersham Pharmacia Biotech) labeled LM probe was added (Hybspeed RPA, Ambion) to 5 mg samples of total RNA from each group together with 45 mg of yeast RNA. For assays on material prepared from animals at stages PM1 or younger, 2 × 10⁴ cpm of probe was used for each reaction. At PM2, 4 × 10⁵ cpm of probe was used to ensure probe excess. Probe was transcribed from Bluescript plasmid containing the F3-500 LM clone linearized with *NotI* (as per Catz et al., 1992). The ³²P-CTP (Amersham No. 20382) labeled probe was transcribed with T7 RNA polymerase (Maxiscript kit; Ambion).

Developmental Expression of LM

To determine the onset of LM expression, whole larynges of males and females were examined at tadpole stages 54, 60, 63, and 66 and at juvenile stages PM0 (1 month PM), PM1

(3 months PM), and PM2 (6 months PM). Since the ratio of cartilage to muscle may differ in the sexes, laryngeal muscle was isolated from PM2 male and female larynges and levels of LM expression were determined. This experiment was done twice.

Prolactin Radioimmunoassays (RIAs)

The pericardium was removed and blood was collected directly from the ventricle in a heparin coated microhematocrit capillary (VWR Scientific) under anesthesia with MS-222. Negative pressure was applied with a mouthpiece connected to the capillary with a length of tubing. Plasma was then obtained by centrifugation at 4000 rpm for 15 min. Plasma from four to six animals was pooled for each PM1 sample, and from three to four animals for each PM2 sample. Samples were placed on ice until centrifuged, and the plasma was then collected and lyophilized for transport.

Isolation of *Xenopus* prolactin (xPRL) and production of antibodies to xPRL are described in Yamashita et al. (1993) and Yamamoto et al. (1995), respectively. The radioiodination of xPRL was accomplished with a modified lactoperoxidase method as described previously (Yamamoto and Kikuyama, 1982; Yamamoto et al., 1989).

A double-antibody method was used in the RIA. The antiserum to xPRL exhibited the ability to bind the radioligand when 100 μ L diluted antiserum and 100 μ L labeled xPRL (approximately 20,000 cpm) were added to each incubation tube containing 300 μ L 1% BSA-PBS [0.01 M phosphate buffer containing 0.14 M NaCl, 1% bovine serum albumin (BSA), and 0.1% NaN₃, pH 7.5]. The RIA was carried out in disposable polystyrene tubes. Standard hormone and plasma were serially diluted with 1% BSA-PBS and added to each assay tube containing 200 μ L 1% BSA-PBS. Each preparation was assayed in duplicate. The xPRL antiserum was diluted 1:5000 (final dilution of 1:25,000) with 0.05 M ethylenediaminetetraacetic acid (EDTA)-PBS (0.01 M phosphate buffer containing 0.14 M NaCl and 0.05 M EDTA, pH 7.5) added with 1% normal rabbit serum. One hundred microliters of diluted antiserum and 100 μ L labeled xPRL diluted with 1% BSA-PBS were added to each tube. All assay tubes were incubated for 24 h at room temperature. After incubation, the immune complexes were precipitated by the addition of 200 μ L 1:100 dilution of goat anti-rabbit immunoglobulin G (IgG) serum with 0.05 M EDTA-PBS containing 3.2% polyethylene glycol 6000. After incubation for 24 h at room temperature, each tube was centrifuged at 10°C for 30 min at 4000 rpm and the supernatant was aspirated. The radioactivity in each precipitate was counted in an Aloka Auto Well Gamma System 600 (Aloka, Tokyo). The radioactivity in tubes containing the antiserum and labeled xPRL, but no unlabeled hormone, was designated 100% and the counts in other tubes were expressed as a fraction of this. When the antiserum was used at a final dilution of 1:25,000, a 40% specific binding of the added radioligand was obtained in the absence of unlabeled prolactin.

Hormonal Manipulations

Dihydrotestosterone (DHT) (5α -androstane-17 β -ol-3-one; Sigma) implants were prepared by mixing silastic polymer

(Dow Corning Silastic silicon type A) with hormone (4:1). This mixture was extruded through a disposable syringe tip to produce a rope of constant width. After overnight curing, the rope was cut into segments containing 0.5 mg of hormone. In all hormonal manipulation experiments, male and female larynges were pooled (except the hypophysectomy of PM2 males). Assignment to sex is difficult in newly postmetamorphic animals as the gonads appear undifferentiated to visual inspection. We thus pooled males and females after conducting a pilot study which established that juvenile males and females respond to androgen treatment equally. In this pilot experiment, stage 66 animals were treated with DHT pellets for 1 day, after which larynges were collected. Larynges were put in separate tubes until each animal's sex was determined by histological analysis of gonads. Larynges were then separated according to sex and LM expression was measured as above. Both males and females expressed equal levels of LM in response to androgen; thus, the proportion of males to females in treatment groups should not have affected LM levels.

Midmetamorphic tadpoles (stage 63) and newly metamorphosed froglets (stage 66) were treated with DHT to examine androgen effects on LM expression. Stage 63 and 66 animals were briefly anesthetized using hypothermia and a pellet was implanted into the dorsal lymph sac through a small incision in treated animals. Animals were DHT treated for 1 day. Twenty larynges from stage 63 tadpoles were pooled in both untreated and treated groups. At stage 66, 15 larynges were pooled for each group. This experiment was done twice.

Since prolactin itself blocks progression through metamorphosis (Tata et al., 1991) and would thus interfere with laryngeal development, we examined interactions between prolactin and DHT at stage 66, when metamorphosis is complete. Four treatment groups were examined: vehicle injected, vehicle injected plus a DHT pellet, prolactin injected, and prolactin injected plus a DHT pellet. DHT pellets were implanted 1 day before sacrifice; animals were injected with prolactin or vehicle 1.5 h before sacrifice. Prolactin (Sigma; ovine) was prepared as a 0.5-mg/mL solution in 10% EtOH. Tadpoles were anesthetized using hypothermia and injected with 25 μ g prolactin in 50 μ L or with vehicle (10% EtOH). Each group contained 15 pooled larynges. This experiment was done three times.

If prolactin is required for induction of LM during early postmetamorphic stages, hypophysectomy should reduce LM expression relative to controls that had received a sham operation. Four treatment groups were prepared: sham hypophysectomized and sham gonadectomized, sham hypophysectomized and gonadectomized plus a DHT pellet, hypophysectomized and gonadectomized plus a DHT pellet, and hypophysectomized and gonadectomized plus a DHT pellet and prolactin injections. Surgery was performed and treatment begun at stage 66. Animals were injected with prolactin (group 4) or vehicle (groups 1–3) daily for 1 month and sacrificed, and larynges were collected as described above. Each group included 15 pooled larynges. This experiment was done twice.

To determine whether the requirement for prolactin secretion is maintained into early juvenile stages, PM2 males

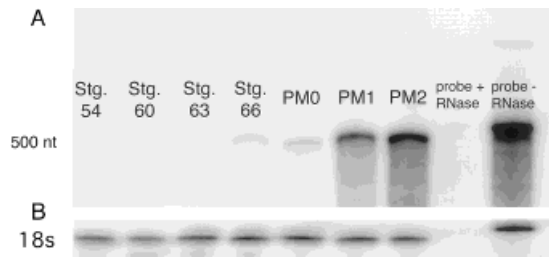


Figure 1 (A) Comparison of LM expression during metamorphosis and early postmetamorphic development. Whole larynges were homogenized and total RNA was used in an RPA with antisense LM probe. This pattern of expression was confirmed with independently isolated RNA samples for each age group. (B) Equivalent amounts of total RNA were assayed for 18s ribosomal RNA.

received either a sham operation or hypophysectomy. After 1 week, larynges (eight from each group) were collected as described above.

If prolactin is required to masculinize the contractile properties of the larynx, depriving frogs of prolactin should impede androgen's ability to change muscle fiber type, a characteristic that can be determined by measuring the percent transient tension at rapid rates of nerve stimulation in an isolated larynx preparation (Tobias and Kelley, 1987). Three treatment groups were prepared: sham hypophysectomized and gonadectomized plus a DHT pellet, hypophysectomized and gonadectomized plus a DHT pellet, and hypophysectomized and gonadectomized plus a DHT pellet and prolactin injections. Surgery was performed and treatments begun at stage 66. Animals were injected with prolactin (group 3) or vehicle (groups 1 and 2) daily for 1 month and sacrificed, and larynges were collected to measure tension properties as described below. This experiment was done twice.

Electrophysiology

All laryngeal measurements were obtained from isolated larynges, a preparation in which electromyogram (EMG) and tension characteristics associated with mate calling have been defined (see Tobias and Kelley, 1987, for a full description of recording and stimulating procedures). Frogs were anesthetized by immersion in 0.1% MS 222. Larynges were removed and maintained in saline throughout the experiment. The laryngeal nerve was stimulated via a suction electrode. Stimulus trains were designed to emulate the male mate call (200-ms duration at 66 Hz) and were separated by 5-s intervals to avoid temporal summation or adaptation. The tendon which connects laryngeal muscles with the sound-producing cartilaginous disks was connected to a force transducer (Grass FT-103) to measure isotonic muscle tension.

Tension recordings were acquired with MacLab (ADInstruments) and used to measure the percentage of transient tension, which represents the relative proportion of transient to maintained tension during stimulus trains delivered to the

nerve. The percentage of transient tension is obtained by dividing the amplitude of the transient portion of the tension record by the amplitude of the total tension and then multiplying by 100 (as in Tobias et al., 1991a).

RESULTS

LM Is Expressed Weakly during Metamorphosis and Strongly after Metamorphosis

Ribonuclease protection assays reveal an ~500-nt protected fragment in laryngeal extracts [Fig. 1(A)]. As expected, the undigested probe was ~50 nt longer than the protected fragment (Fig. 1, final lane). We used RPA instead of *in situ* hybridization because of the high sequence homology among sarcomeric myosins which would have resulted in cross-hybridization of the LM probe to other myosin heavy chain RNAs (Nguyen et al., 1982). To confirm that LM expression is specific to the larynx, RPAs were also carried out using RNA from leg and arm muscles. No LM transcript was detectable in either (data not shown). RPAs using 18s rRNA probe revealed equivalent RNA loading in each lane [Fig. 1(B)].

The level of expression of LM increased with age (Fig. 1). Under these hybridization conditions, no LM expression was detectable at tadpole stage 54 (~26 days pf). By stage 60 (46 days pf), a transcript was detectable; expression at tadpole stage 66 (58 days pf) was somewhat higher than at earlier stages; this level of expression was maintained into the immediate postmetamorphic period (PM0; 1 month PM). At PM1 (3 months PM), however, the level of LM expression dramatically increased relative to earlier stages and further increased at PM2 (6 months PM).

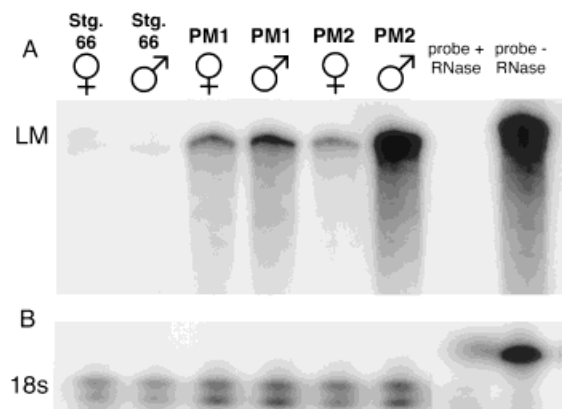


Figure 2 (A) Comparison of LM expression in males and females during early postmetamorphic development. (B) Equivalent amounts of total RNA were assayed for 18s ribosomal RNA.

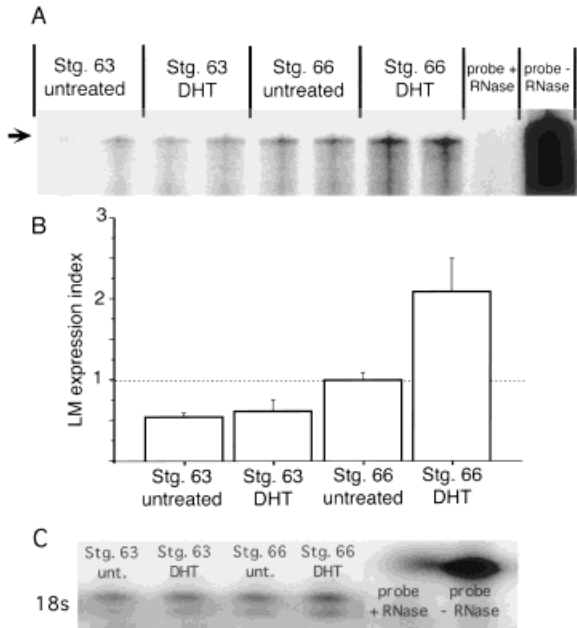


Figure 3 (A) Response of LM expression to DHT treatment at metamorphic climax (Stg. 63) and shortly after metamorphosis (Stg. 66). Duplicate RPAs were carried out for each treatment group. (B) Mean expression level of LM as measured by densitometric analysis. Error bars indicate S.E.M.s of triplicate RPAs. (C) Equivalent amounts of total RNA were assayed for 18s ribosomal RNA.

Expression of LM Is Sexually Dimorphic in Juveniles

At the end of metamorphosis (tadpole stage 66), LM expression in larynges was equivalent in the sexes (Fig. 2). By PM1 (3 months PM), however, expression was higher in males than in females; densitometric analyses of autoradiograms revealed that male expression was 1.6 times that in the female. At PM2, the male/female difference was more striking; densitometric analyses indicated that laryngeal LM expression was 10 times higher in males. When LM expression in muscle was examined separately from cartilage, the ratio of LM expression in males and females was equivalent to that observed in the entire larynx (data not shown); possible sex differences in muscle/cartilage ratios thus do not account for results obtained.

Prolactin and Androgen-Regulated LM Expression

Androgen's ability to elicit LM expression at metamorphic climax and at the end of metamorphosis was compared. At tadpole stage 63, administration of androgen (DHT) for 24 h had little effect on LM expression (Fig. 3). By stage 66, however, LM expres-

sion was up-regulated two-fold in larynges from DHT-treated animals compared to untreated controls.

During this same period, the prolactin gene is also expressed at high levels in the *Xenopus laevis* pituitary (Buckbinder and Brown, 1993). Does prolactin affect LM expression? Since prolactin itself blocks progression through metamorphosis (Tata et al., 1991) and would thus interfere with laryngeal development, we examined interactions between prolactin and DHT at stage 66, when metamorphosis is complete. Prolactin treatment alone did not up-regulate LM expression (Fig. 4). However, prolactin treatment enhanced DHT-induced LM expression. When administered at stage 66, prolactin treatment produced a fourfold increase in DHT induction of LM expression compared to DHT-treated vehicle-injected controls. Prolactin potentiation of androgen regulation was observable 1.5 h after treatment. We conclude that prolactin potentiates DHT-induced increases in LM expression.

If prolactin is required for induction of LM during early postmetamorphic stages, hypophysectomy should reduce LM expression relative to controls that had received a sham operation. Hypophysectomy at stage 66, which removes the source of endogenous prolactin, markedly reduced DHT-induced LM expression by PM0 (Fig. 5). Daily prolactin injections restored DHT-

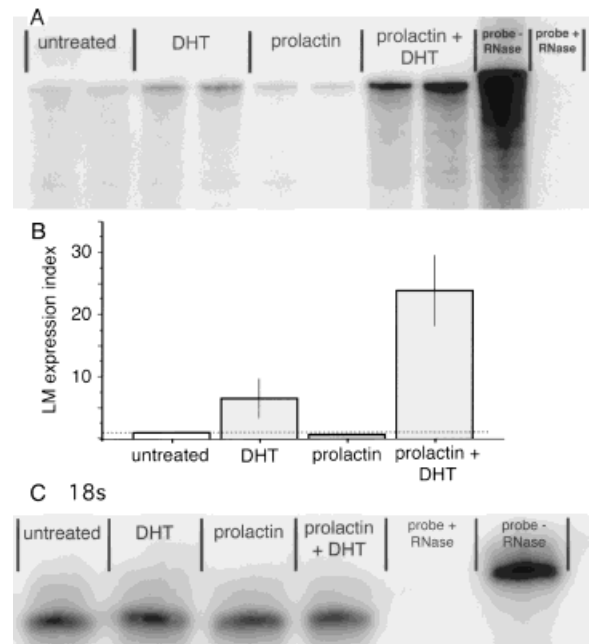


Figure 4 (A) Mean LM expression induced by DHT and prolactin shortly after metamorphosis. Duplicate RPAs were carried out for each treatment group. (B) Expression levels as measured by densitometric analysis. Vertical lines indicate the range of values of two independent determinations. (C) Equivalent amounts of total RNA were assayed for 18s ribosomal RNA.

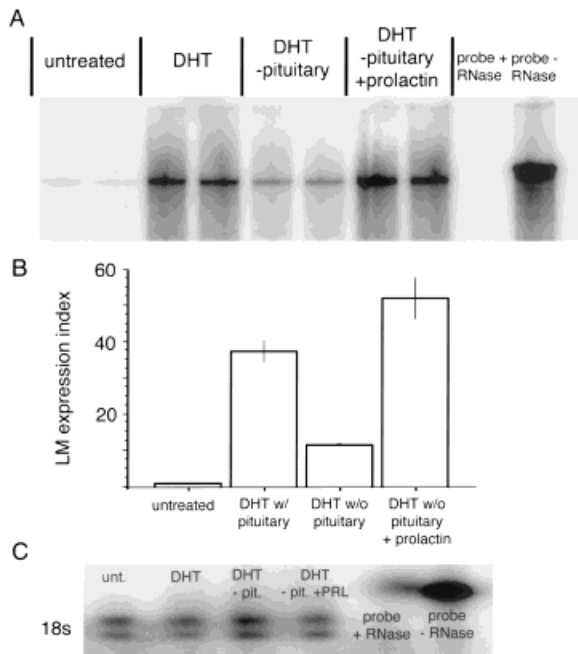


Figure 5 (A) LM expression at PM0 (1 month postmetamorphic) after 1 month of DHT treatment with pituitary intact, pituitary removed, and pituitary removed with prolactin injected daily. (B) Expression levels measured by densitometric analysis. Vertical lines indicate the range of values of the two RPA reactions from each group. (C) Equivalent amounts of total RNA were assayed for 18s ribosomal RNA.

induced LM expression. However, the continued presence of the pituitary is not required for continued LM expression. Hypophysectomy at PM2 (6 months PM) had no effect on LM expression (measured 1 week after surgery) in larynges of gonadally intact males (Fig. 6). RIA was performed to confirm that prolactin had been effectively reduced in hypophysectomized animals (Table 1). To determine whether different levels of prolactin are responsible for the difference in LM expression in males and females, RIA was used to measure circulating prolactin levels at PM1 and PM2. No significant difference in prolactin levels was apparent (Table 1).

Effect of Prolactin on Contractile Characteristics of Laryngeal Muscle

To show that prolactin secretion contributes to the masculinization of contractile characteristics of laryngeal muscle, we measured the production of tension transients (which reflect muscle fiber transformation from slow to fast) after androgen treatment with and without prolactin (Fig. 7). After a month of androgen treatment which began at the conclusion of metamorphosis, the animals that had received DHT implants averaged 89% transient tension ($n = 7$), whereas

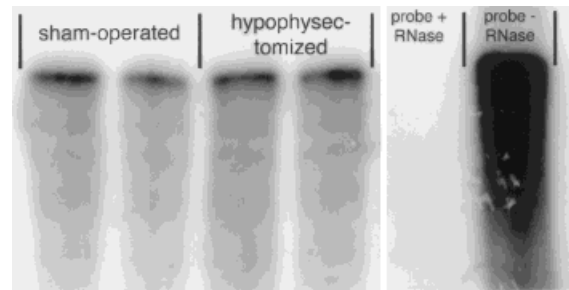


Figure 6 Expression of LM after hypophysectomy at PM2. Duplicate RPAs were carried out for each group, yielding equivalent results.

animals that were DHT treated but also hypophysectomized averaged 60% transient tension ($n = 7$). Replacement of prolactin in hypophysectomized animals restored androgen's ability to bring transient tension to 85% ($n = 8$).

DISCUSSION

Prolactin Secretion Opens Sensitive Period for Androgen-Induced LM Expression

The results described above indicate that prolactin secretion opens the sensitive period for androgen-induced LM expression. LM is weakly expressed in the larynx until after metamorphosis is complete, although androgen and androgen receptor are present from much earlier stages (Kang et al., 1995; Cohen and Kelley, 1996). In addition, expression of LM is largely unaffected by androgen when administered at stage 63, but increases twofold when DHT is administered at stage 66. This change in sensitivity coincides with prolactin expression patterns during metamorphosis, very low at stage 63 and peaking at stage 66 (Buckbinder and Brown, 1993). Removal of the pituitary markedly reduces androgen-induced LM expression, which is restored by prolactin treatment. Thus, exogenous prolactin can rescue androgen-reg-

Table 1 *Xenopus laevis* Prolactin Levels (ng/mL Plasma)

	Males	Females
PM1	13.42	13.32
PM2	12.40	8.55
HX PM2	2.14	1.32

The value is the average of two determinations of plasma pooled from four to six animals (PM1) and three to four animals (PM2). HX = hypophysectomized.

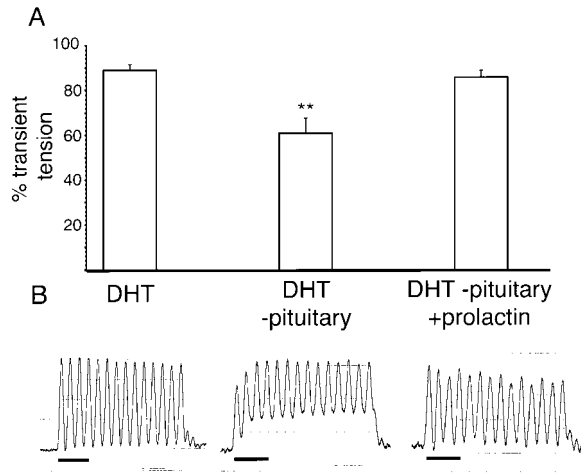


Figure 7 (A) Percent transient tension produced by laryngeal muscle at PM0 after 1 month of DHT treatment with the pituitary intact ($n = 7$), without the pituitary ($n = 7$), and without the pituitary but with daily prolactin injections ($n = 8$). $**p < .005$ for comparisons of DHT-pituitary with the other two groups (Mann-Whitney test). (B) Samples of muscle tension traces from each group. Scale bars = 50 ms.

ulated LM expression in the absence of the pituitary. While prolactin treatment augments androgen-induced LM expression, no effect is seen with prolactin alone. This observation indicates that prolactin's effect on LM expression is androgen dependent. Prolactin has an effect on the contractile properties of the larynx as well. Removing the pituitary reduces the ability of androgen to change the laryngeal muscle from the low transient tension typical of immature males to the high transient tension typical of adult males (Tobias et al., 1991a). Daily prolactin injections restore androgen's ability to induce transient tension in the absence of the pituitary. Taken together, these data implicate prolactin as a necessary factor in the development of masculine muscle twitch type, a necessary transformation which underlies the male's ability to produce his courtship songs.

Successive Aspects of Masculinization of Laryngeal Muscle Are Initiated by Different Hormones

Early in myogenesis, myoblasts proliferate in response to mitogenic factors. After these myoblasts become postmitotic myocytes, they begin to express myosin heavy chain genes before they fuse and become multinucleated myotubes. During metamorphosis, the larynx must first be exposed to thyroid hormone before androgen is able to elicit myoblast and chondroblast proliferation (Cohen and Kelley, 1996). The developmental sequence that leads to masculin-

ization of the laryngeal muscle by androgen therefore seems to be as follows. Exposure to thyroid hormone is essentially the initiating event, since it allows androgen to induce cell proliferation in the larynx during metamorphic climax. In addition, thyroid hormone indirectly affects LM expression by inducing prolactin synthesis (Buckbinder and Brown, 1993). Prolactin then synergizes with androgen to induce LM expression. Thyroid hormone's control of both laryngeal cell proliferation and prolactin secretion allows precise timing of these sequential events in muscle masculinization. One possibility is that when laryngeal myoblasts become postmitotic myocytes, they lose their androgen sensitivity and prolactin resensitizes them to androgen, thus allowing masculinization of twitch type.

Prolactin and Androgen's Role in Development of Secondary Sexual Characteristics

A hormone may be needed merely to initiate the development of a secondary sexual characteristic, such as the expression of LM, or it may be needed to both initiate and maintain that characteristic. The synergy between androgen and prolactin has been demonstrated in several species affecting both development of secondary sexual characteristics and induction of sexual behavior (Toyoda et al., 1993). During the breeding season of the red-spotted newt, the tail fin of the male grows and the nuptial pads form. The tail fin height and nuptial pads can be maintained beyond the breeding season by prolactin and androgen treatment (Singhas and Dent, 1975). In this system, prolactin is needed for both the induction and maintenance of secondary sexual characteristics, unlike the larynx where induction but not maintenance requires prolactin.

Not only do prolactin and androgen work together to induce the development of body structures used for courtship, they also facilitate the development of sexual accessory glands. Prolactin and androgen together have been shown to induce development of cloacal glands in the juvenile red-bellied newt and tiger salamander (Kikuyama et al., 1975, Norris et al., 1989). The lateral gland of the cloaca is responsible for creating spermatophores, packets of semen deposited in the female during fertilization (Noble, 1954). The abdominal gland of the cloaca secretes a female-attracting pheromone (Kikuyama et al., 1995). Combined testosterone and prolactin treatment resulted in significant growth and induction of secretion of the lateral gland and elevation of the pheromone content in the abdominal gland (Yamamoto et al., 1996). Similar results have been shown in mammals. Treat-

ment of juvenile rats or Indian palm squirrels with testosterone and prolactin causes growth and stimulation of secretory activity of the prostate (Grayhack et al., 1955; Reddi, 1969). In meadow voles, combined prolactin and testosterone treatment has also been shown to increase the attractiveness of male odors to females (Ferkin et al., 1997). In all these experiments, prolactin and testosterone together had a significantly greater effect than either hormone administered alone.

Other Genes Influenced by Prolactin and Androgen Synergy: Possible Mechanisms

There are other systems in which both prolactin and androgen are needed for gene transcription. In these systems putative androgen response elements (AREs) have been characterized in the promoter region for the gene, but no prolactin-induced transcription factor response elements have been found. For instance, prolactin and androgen synergize to induce the production of citrate in the mammalian prostate. mAAAT is the enzyme which transforms aspartate into a citrate precursor (for review, see Costello and Franklin, 1994). Prolactin and androgen have both been shown to induce expression of the mAAAT gene (Franklin et al., 1992). Recent data show that the promoter region of the mAAAT gene contains putative androgen response elements (Juang et al., 1995). Another gene shown to be androgen and prolactin sensitive is the prolactin-inducible protein (PIP)/gross cystic disease fluid protein (GCDFF-15), which is present in human breast tumors. Its promoter has putative AREs as well (Mylal et al., 1991). Regarding the mechanism of prolactin's influence on androgen responsive genes, some evidence indicates it may be through the androgen receptor itself. Prolactin treatment has been shown to cause greater nuclear localization of androgen receptor in the lateral prostate gland (Prins, 1987). If prolactin activates transcription solely by activating the androgen receptor, it would explain the absence of an exclusively prolactin-induced transcription factor binding site on prolactin plus androgen-responsive genes. It is still possible, however, that prolactin-induced transcription factor binding sites exist on these genes and have not been identified.

Possible Mediators of Synergy between Prolactin and Androgen Receptor

The effects of prolactin on androgen-responsive genes may be mediated by PKC. PKC has been shown to synergize with androgen to activate expression of reporter genes through the AREs of the MMTV promoter (de Ruiter et al., 1995). PKC has also been

shown to allow androgen-induced expression of the mouse vas deferens specific aldose reductase-like protein (Fabre et al., 1996). In addition, PKC has been shown to mediate the effect of prolactin on β -casein expression in mammary cells (Marte et al., 1994). Prolactin activation of PKC may mediate prolactin's effect on AR activation.

Prolactin Is Integral to Masculinization in Vertebrates

This study demonstrates that prolactin plays an integral part in the masculinization of the laryngeal muscle of *X. laevis* by opening the sensitive period for androgen-induced LM expression. Stringent endocrine-mediated temporal control of androgen sensitivity is important to the developing frog, as it delays sexual differentiation until after metamorphosis is complete.

In the past, androgen was thought to be solely responsible for the process of masculinization. We now understand that masculinization is instead the result of a complex set of interactions between many hormones. This interaction adds to the multiple ways in which androgen regulation of target gene expression can be modulated. The role of prolactin, usually associated with the development of female sexual characteristics and behavior, can be expanded to include development of male sexual characteristics.

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