Androgen receptor expression and sexual differentiation of effectors for courtship song in *Xenopus laevis*

L.M. Fischer and D.B. Kelley

Sexually differentiated behaviors in vertebrates result from sex-specific patterns of hormone secretion during development and in adulthood. To investigate the molecular basis of sexually differentiated behaviors, we are studying the courtship song of the African clawed frog, *Xenopus laevis*. Male mate calling behavior is produced by activity of a circuit in the central nervous system and by the vocal organ, the larynx. Both are strikingly different in males and females. These differences are due to alterations in the developmental programs of brain and larynx as a result of androgen secretion from the testes. Steroid hormones produce their effects by binding to receptor proteins, which act as ligand-activated transcription factors. In clawed frogs, androgen receptor mRNA is strongly and specifically expressed in nuclei of the vocal circuit and tissues of the larynx. The larynx expresses 9.6 and 8.0 kb androgen receptor mRNAs; high levels of androgen receptor mRNA and the presence of the 8.0 kb transcript are associated with androgen-inducible cell proliferation.

**Key words:** cell proliferation / larynx / mate calling behavior / sexual dimorphism / steroid

A central problem in this field is to understand how hormones control the expression of sexually differentiated behaviors. Steroid hormones exert their genomic effects directly through specific receptor proteins that are nuclear transcription factors, which, when bound to steroid hormones, can enhance or repress the transcription of specific gene products. Thus steroid hormones can control differentiation by altering the tissue repertoire of expressed genes. The specific genes involved and the cascade of molecular and cellular events that follow hormone binding to the receptor are poorly understood.

We have studied hormonal regulation of behavior in a highly tractable experimental system, the sexually differentiated courtship song in the African frog, *Xenopus laevis*. Song is produced by the vocal organ, the larynx, in response to activity in a central nervous system (CNS) pathway. Laryngeal muscles and innervating motor neurons comprise a neuro-muscular system dedicated exclusively to vocalization. Pronounced sex differences characterize neural and muscle effectors for vocal behavior and many of these differences appear to be under hormonal control. Activity in the CNS vocal circuit requires androgen secretion in adulthood. The development of the vocal organ depends on androgen, which stimulates proliferation of muscle and cartilage precursor cells in juvenile larynges and is also necessary for cell differentiation underlying the adult masculine phenotype. Using cloning and gene expression techniques, we have shown that during periods of androgen-directed sexual differentiation, neurons in the CNS vocal circuit and cells of the larynx express high levels of androgen receptor mRNA (M. Cohen, personal communication; ref 7). Within the larynx, undifferentiated cells express the highest levels of androgen receptor transcripts. In response to hormone, these cells are invoked to proliferate and differentiate, whereupon their androgen receptor mRNA expression levels decrease.

Many stereotyped courtship and mating behaviors are exhibited only by one sex and so are termed sexually differentiated or dimorphic. How are sex-specific behaviors produced? Sex differences in vertebrate behaviors are primarily controlled by the secretion of gonadal steroids: androgens from the male testis and estrogen and progesterone from the female ovary. When the ontogeny of neurons and muscle effectors involved in reproductive behavior is linked to sex-specific patterns of hormone secretion, the result is a sexually differentiated program of development leading to sexually dimorphic behaviors in adulthood. Sex differences in behavior can also result from differing hormonal secretions in adult life.

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Vocal behavior in *X. laevis*

Male and female *X. laevis* communicate their reproductive state with specific vocalizations. During the breeding season, male frogs sing or 'mate call' to attract fertile and receptive females. When clasped, sexually unreceptive females 'tick' to repel males. Male frogs will also tick when clasped by another male. Mate calling ability is, however, confined to sexually mature males; females cannot sing. Ticking and mate calling differ in trill rate, complexity and amplitude modulation. Mate calls consist of rapid trills with alternating slow (30 Hz) and fast (60 Hz) phases, with the fast portion of the mate call becoming progressively louder. Ticking is a slow (6 Hz), monotonous trill.

Secretion of testicular androgen is required to activate the neural circuitry underlying male song. Levels of circulating androgen are higher in males than in females in adulthood. Castrated males do not mate call even though their vocal organ is capable of producing the requisite trills. Because castrated males do sing when resupplied with androgen, we infer that androgen activates the neural pathway for song. Adult females cannot sing even when given exogenous androgen, indicating that the effectors underlying song production are sexually differentiated. What about the female capacity for song? A female given a testis implant early in post metamorphic life can produce completely masculine mate calls (J. Watson, personal communication). Although females implanted later in development can also mate call, acoustic and temporal properties of their song are aberrant, attributable at least in part to incomplete masculinization of the larynx. These results indicate that females retain some potential for vocal system masculinization which diminishes with age (J. Watson, personal communication). Of the androgens we have studied, the 5α-reduced metabolite of testosterone (5α-dihydrotestosterone, DHT) appears to be the most effective in masculinization of the larynx and activation of the behavior in adulthood.

Despite extensive investigation, we have not yet identified a role for estrogen secretion or conversion of androgen to estrogenic metabolites in this system. The larynx and the laryngeal motor neurons do not contain estrogen receptors. The purely androgenic control of the song system in *X. laevis* is similar to androgenic control of sexually dimorphic neuromuscular systems in songbirds and in rats (refs 12, 13; and see Forger and Breedlove, this issue).

Sex specific development of the *X. laevis* song system

The brain

Laryngeal muscles contract to produce calls in response to activity of the laryngeal motor neurons located in cranial nerve nucleus IX-X in the caudal medulla. Using retrograde labelling techniques, we have outlined an anatomically connected system of brain nuclei that provides input to the calling motor neurons (ref 15; see Figure 1). Based on our studies in *X. laevis* and those in other anurans, we believe that the CNS motor pathway for song includes a vocal pattern generator in the superior reticular formation (the pretrigeminal nucleus of the dorsal tegmental area of the medulla, or DTAM), interneurons within the motor nucleus (N.) of IX-X and in the inferior reticular formation, sensory nuclei in the thalamus (auditory, lateral line and somatosensory) and certain nuclei of ventral diencephalon (anterior preoptic area) and telencephalon (ventral striatum).

Portions of the adult vocal circuitry in the brain differ in the sexes. Males have a robust connection from N.IX-X interneurons to DTAM and a projection from preoptic area to DTAM that females lack. Interneurons in N.IX-X of males have markedly longer dendrites than females and males have twice as many laryngeal motor neurons and axons as do females.

Sex differences in the neural circuitry underlying song in *X. laevis* are thus quite dramatic. How do they originate? In vertebrates, sexual dimorphism of brain regions results from differences in development as well as events during adult life. The early development of sexually differentiated brain nuclei is the same in males and females. In males the developmental program of the CNS is then altered as a result of gonadal hormone secretion. Some effects of hormone exposure (termed organizational) are permanent—even if never again exposed to hormone, the structure or function of the hormone-sensitive brain nucleus is irrevocably altered. Other sex differences in the adult brain are not permanent but wax and wane with the levels of circulating hormones; such effects of steroids are termed activational.

Which of the marked sex differences in the CNS pathway for courtship song behavior in *X. laevis* are due to organizational effects of testicular androgen exposure and which are due to activational effects?
Sex differences in number of laryngeal motor neurons represent the organizational effects of androgen on this system. Adult males maintain more motor neurons than females even when castrated (D. Kelley, unpublished observation). Counts of axons entering laryngeal muscle\textsuperscript{18} demonstrate that axonal projections of motor neurons are the same in males and females during early tadpole stages but diverge during metamorphosis, a period spanning late tadpole stages 54-66.\textsuperscript{19} At tadpole stage 56, when gonadal differentiation is first evident,\textsuperscript{19} the number of axons entering laryngeal muscle is the same in males and females.\textsuperscript{17} In males but not in females there is a wave of axon addition between stages 59 and 62. Between stages 62 and 66 both sexes lose laryngeal axons. Adult values for axon numbers are attained during postmetamorphic development. Treatment with flutamide, an anti-androgen, between stages 54 and 62 reduces axonal outgrowth from male laryngeal motor neurons, whereas female axon numbers are not affected (J. Robertson, personal communication). In both sexes, dihydrotestosterone increases axon numbers. Together these lines of evidence indicate that the sexual differentiation of laryngeal motor axons is due to testicular androgen secretion during late tadpole development.

The restoration of mate calling in castrated adult males represents a purely activational effect of androgen in this system. We know that the larynx of a castrated adult male remains capable of song for up to two years;\textsuperscript{3} exogenous androgen can activate the neural circuitry for mate calling in these males. The connections of brain nuclei that participate in vocal behavior differ markedly in adult male and female \textit{X. laevis}.\textsuperscript{15} It is possible that connections between song nuclei in the brain are highly plastic and underlie the activational effects of androgen on calling. Alternatively, the connectivity in song nuclei characteristic of males may represent an organizational effect of androgen secretion, necessary but not sufficient for mate call production.

\textit{The larynx}

The adult male larynx is a hollow cartilagenous box composed of two plates of hyaline cartilage, seamed together by elastic cartilage and flanked on either
Figure 2. Schematic representation of adult male and female larynges shown in horizontal section. The larynx is a hollow box constructed of hyaline cartilage (hc). Sheaths of bipinnate muscle (m) overlie the cartilaginous skeleton and insert bilaterally via a tendon into the sound-producing elements, the arytenoid discs (ad). Male larynges contain an additional tissue type, elastic cartilage (ec), located within the hyaline cartilage and around the arytenoid discs. The anterior portion of the laryngeal muscle (coarse shading) also lies adjacent to the arytenoid discs.

The sex difference in vocal ability is attributable in part to the sexually differentiated larynx. The structures of adult male and female larynges differ strikingly (Figure 2). The male larynx is 2-3 times larger and is more box-like in structure due to an expansion of muscle and cartilage; males have 1.7 times as many laryngeal muscle fibers as do females. Laryngeal muscle fiber type is predominantly slow twitch in females. In males it is entirely fast twitch, a property that enables the male muscle to produce very rapid transient tension spikes and the rapid trills required for mate calling. The hyaline cartilage morphology in the adult male larynx is complex relative to the female.
Males also have elastic cartilage, which is completely missing in female larynges.

At the end of metamorphosis (PM stage 0; Figure 3), male and female larynges are morphologically similar, and muscle properties and cartilage composition are the same in both sexes. Cellular characteristics of the larynx begin to masculinize progressively during the postmetamorphic (PM) period; first muscle fiber number (PM stage 1), then elastic cartilage differentiation (PM stage 2) and finally the ability of muscle to produce transient tension (PM stage 3) due to fiber type switching. Elastic cartilage completes masculinization relatively late (PM stage 5). At earlier stages, the precursors of this tissue comprise a zone of undifferentiated mesenchymal cells at the site of the future elastic cartilage. In females, this cell population does not differentiate and persists into adulthood. The female larynx continues to grow in proportion to body size during the entire lifespan of the animal and the undifferentiated mesenchymal cells may contribute to this growth by providing hyaline cartilage precursor cells.

Masculinization of the larynx requires secretion of testicular androgen. Levels of circulating androgens (testosterone and dihydrotestosterone) are greater in males than in females during postmetamorphic development (L. Lambdin, personal communication). Castration arrests postmetamorphic differentiation of muscle fiber number and twitch properties. Treatment with androgen reinstates masculinization in castrated, developing males, and implantation of a testis or of an androgen pellet masculinizes laryngeal properties in females. We have found that a three week exposure to androgen induces the zone of undifferentiated cells to become elastic cartilage both precociously in males and also in females, that would otherwise never develop this tissue type.

We have examined the underlying cellular features of laryngeal differentiation to see why the female larynx loses the ability to become masculinized. Some properties of the larynx can be masculinized in females at any age provided that a very high level of androgen is supplied for an extended period. For example, muscle fiber twitch

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Figure 3. Postmetamorphic stages of development in *Xenopus laevis* showing progressive masculinization of laryngeal characteristics. Laryngeal differentiation in males takes place primarily after metamorphosis. To study laryngeal masculinization, we have divided postmetamorphic (PM) development into 7 stages based on larynx weight, which tightly correlates to degree of vocal system masculinization. These PM stages span the period from the end of metamorphosis (PM stage 0: tadpole stage 66) to adulthood (PM stage 6, 1 to 2 years postmetamorphosis). Stage assignment and representative male larynx weights are given at the top. ♀ indicates when a given trait or response becomes dimorphic between the sexes. ♂ indicates when that trait or response is fully masculinized, i.e. does not differ from that of an adult male. Notice the temporal correspondence of androgen receptor (AR) expression with dihydrotestosterone (DHT)-induced proliferation and differentiation of elastic cartilage.
type can be completely masculinized even in adult females by many months of exposure to supraphysiological levels of androgen. In contrast, in males castrated at metamorphosis, full masculinization can be readily achieved by brief androgen exposure at any time in development. Thus the diminished capacity for masculinization of fiber type in the female reflects a progressive loss of sensitivity to androgen. Other properties of the larynx have a strict limit on when, during development, androgen can act. For example, sensitivity of muscle fiber number to androgen is completely lost in females early in development: when androgen is administered to females at PM stage 0, muscle fiber number is increased; but not increased when it is given at PM stage 2. We do not yet know whether this inability to increase fiber number reflects a restriction on proliferation of the muscle stem cells (myoblasts) or on their ability to fuse and form multinucleated muscle fibers. Laryngeal cartilages of females also seem to lose the ability to respond to androgen early in development.

Androgenic induction of proliferation in the larynx

To organize a fully masculinized larynx, androgen induces cellular proliferation and differentiation during metamorphic development. We have examined cellular sex differences in response to androgen by providing exogenous hormone at various developmental stages. In juvenile (early postmetamorphic) males and females, dihydrotestosterone induces marked proliferation of cells in all compartments of the larynx, including the hyaline cartilage and the muscle. Most strikingly responsive are cells within the undifferentiated mesenchymal zone and the muscle. Later in development, exogenous androgen does not induce cell proliferation in male larynges, whereas significant proliferation is still seen in all tissue compartments of the female larynx, though to a lesser extent than in a younger female. The undifferentiated mesenchymal zone of the larynx is still found in adult females where it remains capable of proliferation and highly responsive to hormones. Much of the loss of the proliferative response during development in males can be accounted for by the loss of the undifferentiated mesenchymal zone, which is replaced by differentiated elastic cartilage. In males these cells are normally exposed to androgen which induces them to proliferate and subsequently differentiate into elastic cartilage. In females they do not proliferate and hence remain undifferentiated due to the absence of sufficient androgen, although exogenous hormone can induce them to follow the male developmental program. The elastic cartilage compartment thus provides a well-defined androgen-sensitive system for examining the molecular basis of androgen-induced proliferation and differentiation.

Evidence for androgen receptor in the vocal system of X. laevis

The brain

The presence of androgen receptor in motor neurons is strongly correlated with a function in male reproductive behaviors. Different species express receptor in different motor neuron pools; in mammals these include spinal motor neurons that control copulatory reflexes (see Forger and Breedlove, this issue) and, in birds, neurons of the hypoglossal nucleus that contribute to song (ref 1; and see Bottjer, this issue). Androgen receptor was first detected in the CNS of X. laevis by administering radioactive androgen (testosterone and dihydrotestosterone) to gonadectomized animals and preparing brain autoradiograms. In the adult brain, androgen binding is largely confined to nuclei implicated in the control of vocal behavior and functions as a specific 'stain' for the calling pathway (Figure 1). Nuclei that comprise the motor pathway for song (laryngeal motor neurons, inferior reticular formation, DTAM) and some sensory nuclei (thalamus, midbrain auditory nuclei) concentrate hormone in both sexes. Labelling is more extensive in N.IX-X of males but males have more laryngeal motor neurons and interneurons than do females. Using receptor autoradiography, labelled cells can be detected in the CNS calling circuit as early as stage 64. At earlier stages, however, it is impossible to castrate the animals and the lack of signal could be due to competition by endogenous hormone. Thus androgen receptor is present during tadpole development in motor neurons that respond to androgen with axonal outgrowth (J. Robertson, personal communication) and in CNS nuclei such as DTAM that are known to have male-specific connections in adults.
The larynx

Our cellular analysis of the progressive masculinization of the larynx provides an opportunity to explore the underlying molecular events, particularly the relation between expression of androgen receptor and responses of laryngeal cells to androgen. The presence of androgen receptor in adult larynx was initially studied by examination of radioactive androgen binding to the cytosolic fraction of muscle homogenates.29 Androgen binding levels are 10-20 times higher in adult male laryngeal muscle than in other muscles and binding levels are 3-4 times greater in male than in female muscle. In whole larynx preparations, adult males exhibit twice as much binding as females.10

Adult levels of androgen binding are achieved by a sexually differentiated program.10 At the end of metamorphosis (PM stage 0), binding activity is very high in both male and female larynges (250-550 fmoles mg⁻¹ protein) and remains high in male larynges over the next 6 months (until PM stage 2), before declining to adult values (40 fmoles mg⁻¹ protein). In females, androgen binding levels decline more rapidly during the postmetamorphic period: 100 fmoles mg⁻¹ protein at PM stage 1, 50 fmoles mg⁻¹ protein at PM stage 3, and 20 fmoles mg⁻¹ protein in adulthood. Levels of androgen binding in males decrease during the developmental period when androgen levels in blood are rising (L. Lambdin, personal communication). If dihydrotestosterone is administered to PM stage 2 animals for 3 weeks, androgen binding levels decline to 20% of their initial value in males and to 50% of the control value in females.10 These studies suggest that levels of androgen receptor in males may be regulated by androgen secretion during development.

Expression of androgen receptor mRNA in the brain

To study the expression of androgen receptor in motor neurons and other components of the vocal pathway in the CNS, we have performed in situ hybridization with the cloned X. laevis androgen receptor as a probe (M. Cohen, personal communication). In adult brain, androgen receptor mRNA was detected in the nucleus of cranial nerves IX-X (laryngeal motor neurons and interneurons), inferior reticular formation, DTAM, the chief sensory nucleus of the Vth cranial nerve, a vestibular nucleus, the laminar nucleus of the torus semicircularis, the anterior pituitary, ventral thalamus and anterior spinal cord. As anticipated, the distribution of androgen receptor-expressing cells parallels previous autoradiographic results using radioactive dihydrotestosterone. The presence of androgen receptor in a vestibular nucleus was only rarely, however, detected in binding studies, so the molecular technique may provide greater sensitivity than previous methods. Currently our studies suggest that there are no differences in location or levels of androgen receptor mRNA expression in male and female brains. Previous studies using tritiated dihydrotestosterone detected androgen-concentrating
Figure 4. Total androgen receptor mRNA expression levels in the larynx during normal post-metamorphic development and after exposure to dihydrotestosterone. Stages are indicated at the top. The cartilage fraction includes the undifferentiated zone of elastic precursor cells. At the end of metamorphosis, PM stage 0, male and female larynges express high and monomorphic levels of androgen receptor mRNA. Males maintain high mRNA expression in laryngeal muscle through PM stage 3, with a slight decrease by adulthood. Females express less androgen receptor message in muscle than males throughout post-metamorphic development and it is only slightly detectable in female laryngeal muscle in adulthood. Within the cartilage component, androgen receptor mRNA expression decreases during early post-metamorphic development for both sexes, though more steeply in males. In adulthood, androgen receptor mRNA expression in the cartilage component is detectable only in females. The white box at PM stage 2 indicates the level of androgen receptor mRNA expression after treatment with dihydrotestosterone (+ DHT) for three weeks.

cells in the CNS from tadpole stage 64 on; no labelled cells were seen at tadpole stage 60. Using in situ hybridization, however, androgen receptor mRNA expression in neurons is detected as early as tadpole stage 56 (M. Cohen, personal communication) and during critical stages when androgen influences axonal outgrowth. We have recently performed a Northern blot analysis, which suggests that the androgen receptor probe used in in situ studies recognizes a 9.6 kb androgen receptor mRNA in adult male brain, a size similar to androgen receptor mRNA in mammals.32,33

Expression of androgen receptor mRNA in the larynx

Unlike the brain, the larynx expresses two transcripts for the androgen receptor, one of 9.6 kb (similar in size to the brain transcript) and one of 8.0 kb. Other tissues in adults (liver, lung, kidney, testes, thigh) express only the 9.6 kb transcript and expression levels are, at most, 1/3 of the levels found in larynx.34 In fact, androgen receptor mRNA expression levels in the larynx are higher than in any other tissue in vertebrates that has been examined.35 Lower molecular weight androgen receptor transcripts have been detected in prostate tumor cell lines and in benign prostatic hyperplasia of humans, although these are not identical in size to the 8.0 kb transcript found in X. laevis larynx.36,37 What is the origin of these two androgen receptor transcripts? Southern analysis of DNA from a homozygous diploid frog using a probe restricted to a single exon suggests that there are two genes for the androgen receptor in X. laevis,36 whereas there is only a single androgen receptor gene in mammals.33 Thus it is possible that the 8.0 kb transcript derives from a second androgen receptor gene. The other possibility is that the second transcript may represent alternatively spliced androgen receptor mRNA, which
must be the case for the second transcript expressed in the hyperplastic prostate.\textsuperscript{37} We will not be able to distinguish among these possibilities until complete genomic and cDNA clones for the \textit{X. laevis} androgen receptor are obtained.

If androgen receptor expression is related to cell proliferation in the larynx, we would expect high levels during early developmental stages when cell number is being established. Androgen receptor mRNA levels during postmetamorphic development in male and female larynges have been studied with Northern analysis.\textsuperscript{7} Whenever possible (from PM stage 2 on), the larynx was separated into muscle and cartilage components. In juvenile males and in females of all ages the cartilage component includes the zone of undifferentiated cells that will give rise to elastic cartilage in males. The developmental expression pattern of the 9.6 kb androgen mRNA is sex specific and is different in laryngeal muscle and cartilage components (Figure 4). A few weeks after metamorphosis (PM stage 1), male and female larynges express extremely high, monomorphic levels of androgen receptor mRNA. Males maintain high levels of androgen receptor message in muscle through PM stage 3 and lower levels in adulthood (PM stage 6). Females express less androgen receptor mRNA in muscle than males throughout postmetamorphic development and barely detectable levels in adulthood. The pattern of expression of the 9.6 kb androgen receptor transcript in the cartilage component differs strikingly from that seen in muscle; it decreases during early post metamorphic development in both sexes, though more steeply in males, and in adulthood is detectable only in the female. Hence the low levels of androgen receptor mRNA characteristic of an adult female may underlie the progressive loss of her potential for masculinization.

The androgen receptor 8.0 kb transcript is also expressed in a sex and tissue specific manner: it is never detected in the absence of the 9.6 kb transcript and seems consistently to be expressed at lower levels. Its maximum expression is seen in juvenile (PM stage 1) whole larynges and in the laryngeal cartilage component of both sexes at PM stage 2; this transcript is absent from laryngeal muscle of older females and from both muscle and cartilage of the adult male. Comparing these data with previous studies on developing larynx, we observed that the 8.0 kb transcript is expressed preferentially during stages when particular tissue types can be induced to proliferate with androgen.\textsuperscript{6,11} Furthermore, we believe that expression of this transcript may permit cells to undergo this response (see below).

Using \textit{in situ} hybridization, androgen receptor message has been detected in the larynx as early as stage 56, when it is also detected in the brain.\textsuperscript{7} At the end of metamorphosis (PM stage 0), male and female larynges express equivalent amounts of androgen receptor mRNA, suggesting that early in development male and female larynges are equally capable of responding to androgen by masculinization. In juveniles, the highest levels of expression occur in the undifferentiated zone of cells located within and lateral to the hyaline cartilage. High levels of message also are detected in the perichondrium (a germinal zone) surrounding the hyaline cartilage. Differentiated hyaline cartilage expresses little or no androgen receptor mRNA. Thus, even though the probe we used recognizes both the 8.0 and 9.6 kb transcripts, the presence of the second transcript in the isolated cartilage preparation can be attributed to the zone of undifferentiated cells and to the perichondrium. The differentiation of these tissue types in males coincides with decreasing androgen receptor mRNA levels (Figure 3). Similarly, androgen receptor mRNA occurs ubiquitously in laryngeal muscle of juveniles, at times when this tissue is populated by muscle stem cells (myoblasts) that have not yet differentiated,\textsuperscript{11} but in older animals, levels decrease concurrent with the conversion of myoblasts to myofibers. Taken together, our data strongly suggest that expression of the 8.0 kb transcript is restricted to mesenchymal stem cells of the larynx that can proliferate and give rise to differentiated muscle and cartilage.

\textbf{Hormonal control of androgen receptor expression}

Autologous regulation is prevalent among steroid receptors.\textsuperscript{38} in mammals, levels of expression of androgen receptor mRNA and protein are known to be regulated by androgen; high titers of androgen downregulate receptor levels, whereas castration raises expression levels, sometimes with a concomitant regression of tissue. During postmetamorphic development in \textit{X. laevis}, males and females differ in circulating androgen levels. Administration of androgen to juveniles decreases hormone binding levels in larynx, suggesting that endogenous androgen may regulate androgen receptor expression during development.\textsuperscript{10}
What happens to androgen receptor mRNA levels in the larynx if this tissue is developmentally arrested by castration or is precociously induced to differentiate by supplying supra-physiological levels of androgen? In post-metamorphic males castration has no effect on androgen receptor mRNA levels; if dihydrotestosterone is given to post-metamorphic frogs for three weeks, laryngeal androgen receptor mRNA levels are downregulated in a sex- and tissue-specific manner (ref 39; Figure 4). The most pronounced changes occur in the cartilage and undifferentiated cells of both sexes; the smallest change is in the female muscle. Thus the decline in androgen binding in response to exogenous hormone can be accounted for at least in part by decreases in androgen receptor mRNA levels.

The ability of androgen to downregulate androgen receptor mRNA expression is related to how much of the 8.0 kb transcript is expressed in a particular tissue component: the higher the expression level of the 8.0 kb transcript, the more dramatic the degree of downregulation. We believe that this finding, together with developmental regulation of androgen receptor mRNA expression in laryngeal cartilage and muscle, can be explained if high levels of androgen receptor mRNA, including the 8.0 kb transcript, are preferentially expressed in stem cells and function to facilitate androgen-induced cell proliferation. As long as the larynx contains large numbers of stem cells, for example, myoblasts and elastic cartilage precursors, androgen receptor transcripts will be present at high levels. When these stem cells differentiate, into myotubes and chondrocytes, the levels of the mRNA, including the 8.0 kb transcript, decline because differentiated cells express only the 9.6 kb transcript at low levels. Indeed the low receptor levels found in differentiated cells may ‘protect’ them by making them refractory to further androgen-stimulated growth. What function might the 8.0 kb transcript play in androgen-regulated cell proliferation? One possibility is that it is more stable than the 9.6 kb form; more androgen receptor protein could be produced from this stable mRNA, enabling cells to maintain the high levels of receptor required for proliferation. Another possibility is that the 8.0 kb mRNA codes for a different androgen receptor than does the 9.6 kb transcript. This novel receptor might bind to a specific androgen and thus control transcription of proliferation-associated genes such as growth factor or mitogen receptors. Further insight into the nature of the molecular machinery underlying the sexual differentiation of the larynx may thus yield important clues as to how hormones regulate cell growth.

**Figure 5. A model for cellular development of the larynx.**

Undifferentiated cells that express high levels of androgen receptor mRNA (shown crosshatched), including a specific 8.0 kb transcript, can respond to androgen with proliferation and consequent differentiation. During proliferation, androgen receptor (AR) mRNA expression is maintained at a high level but as cells differentiate, levels decline. In normal males, rising titers of androgen stimulate this cellular program. In females, due to lack of androgen, stem cells do not proliferate or differentiate and are maintained into adulthood. Females administered androgen will follow a male typical differentiation program. Androgen receptor downregulation seen in response to exposure to dihydrotestosterone (+ DHT) in both sexes may be due to terminal differentiation of responsive stem cells. This effect is permanent and cannot be reversed in males by castration.

**A model for androgen receptor function in vocal system differentiation**

Androgen receptor is expressed in the vocal system of *X. laevis* both in adulthood, when androgen secretion is required to elicit vocal behaviors, and during development, when androgen secretion masculinizes neuroeffector circuits for song. In the differentiated vocal system of an adult male, detectable levels of androgen receptor remain only in the CNS calling circuit and the laryngeal muscle. Androgen receptor function in adulthood may be to activate the neural circuit responsible for the behavior and to maintain the correct twitch properties in the responding muscle.
Androgen receptor is present in the CNS when some of the earliest events in sexual differentiation, such as changes in number of laryngeal motor axons, are taking place. Antiandrogen blocks and androgen stimulates axon outgrowth. It is thus likely that sexual differentiation of neurons is mediated by the complex of androgen with its receptor. In the juvenile larynx, androgen receptor may play an important role in the control of proliferation and differentiation. We hypothesize that androgens induce proliferation of undifferentiated muscle and cartilage precursor cells provided that they express high levels of androgen receptor message, including the 8.0 kb transcript (Figure 5). Consequent differentiation of these cells is accompanied by downregulation of androgen receptor mRNA; decreases in receptor levels may make these cells refractory to further androgen-stimulated cell division. In males, rising androgen titers stimulate cells to divide and later to differentiate. In females, due to lack of androgen, hormone sensitive stem cells do not proliferate and persist into adulthood. The downregulation of androgen receptor that is seen in normal developing males and in both sexes in response to exogenous dihydrotestosterone appears to be due to terminal differentiation of these stem cells. This effect may be permanent because it is not reversed by castration in males.

**References**
