An androgen receptor mRNA isoform associated with hormone-induced cell proliferation

(sexual differentiation/steroid receptor/*Xenopus laevis*/larynx)

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ABSTRACT The larynx of male *Xenopus laevis* undergoes an androgen-driven developmental transformation that enables the adult to produce his complex mate attraction song. During the early postmetamorphic period, androgen directs proliferation and differentiation of laryngeal muscle and cartilage. To explore the cellular and molecular basis of androgen control, we have cloned an androgen receptor cDNA from juvenile larynx. Here we identify two androgen receptor mRNA isoforms, α and β, differing within the A/B, or hypervariable, domain. Northern blot analyses reveal that the β isoform is transiently expressed during early juvenile stages, whereas the α transcript is expressed throughout postmetamorphic life. Using *in situ* hybridization and [^{3}H]thymidine autoradiography, we examined the expression of androgen receptor mRNA isoforms during androgen-evoked cell proliferation and differentiation. The α and β transcripts are coexpressed in proliferating tissues of the juvenile larynx; in postmitotic differentiated tissues, only the α transcript is expressed. Because androgen receptor β mRNA is specifically expressed during hormone-evoked cell proliferation, we propose that this developmentally regulated mRNA isoform is required for the masculine program of cell addition within the developing vocal organ.

In vertebrates, differentiation of the masculine phenotype is directed by secretion of steroid hormones, usually androgens (1). To become masculinized, androgen target tissues must express a functional receptor that acts as a ligand-activated transcription factor (1–3). The androgen receptor (AR) belongs to the steroid hormone receptor gene superfamily (2, 4, 5). For certain members of this family (e.g., thyroxine and retinoic acid), specific mRNA isoforms exist that are transiently expressed during key stages in development (6, 7); the functions of such receptor isoforms are largely unknown.

The mate attraction song of male *Xenopus laevis* is produced by the larynx, an organ whose masculinization is controlled by testicular androgen secretion. At the end of metamorphosis, laryngeal morphology is female-like in both sexes (8). The male larynx then undergoes an androgen-driven program, which results in formation of the complex laryngeal cartilage skeleton and robust musculature required for song production. In male muscle, myoblasts proliferate and then fuse; fiber number increases rapidly (9). In male cartilage, cells proliferate markedly in the perichondrium of the hyaline cartilage and in a zone of precartilage, which later differentiates into elastic cartilage (8, 10). We have shown that proliferation of muscle and cartilage depends on the secretion of testicular androgen and is limited to early juvenile stages (9, 10). The female larynx does not show comparable cell proliferation during these juvenile stages and is incapable of producing male song (11).

The extreme sensitivity of the developing larynx to androgen coincides with high levels of AR expression as demonstrated by hormone binding (12). Using the PCR and laryngeal cDNA, we cloned a fragment of a *X. laevis* AR cDNA containing the DNA and ligand binding domains (5). Northern blot analysis using this fragment detected a 9.6-kb mRNA (AR α mRNA) in testis, kidney, and liver (5); the size and tissue distribution of this transcript are similar to those reported for AR mRNA in mammals (2–5). The highest level of AR α mRNA expression was in the juvenile male larynx where levels exceeded those of adult tissues including testis. In developing larynx, an additional 8.0-kb mRNA (AR β mRNA) was also present; this mRNA was not detected in other tissues examined (5). The present study was designed to determine whether the two forms of AR mRNA present in juvenile larynx are developmentally regulated and to explore the relation between expression of these AR transcripts and androgen-evoked cell proliferation and differentiation.

MATERIALS AND METHODS

Animals. Postmetamorphic (PM) male *X. laevis* approximately 6, 9, and 24 months old were obtained from Nasco (Fort Atkinson, WI). Males were assigned to developmental stages (PM2, -3, or -6, respectively) based on laryngeal weight at sacrifice. These stages (13) correspond to the attainment of milestones in laryngeal masculinization such as a masculine complement of muscle fibers (PM2), differentiation of elastic cartilage (PM3), and the ability to produce full blown male songs (PM6).

Northern Blot Hybridization. Total RNA was extracted from laryngeal muscle and cartilage separately by the RNA-zol method (Tel-Test, Friendswood, TX). Pelleted RNA was solubilized in 4 M LiCl to remove contaminating polysaccharides (14); 10 μg of each sample was denatured with glyoxal (15), loaded on 1.2% agarose gels, and run in 0.01 M phosphate buffer (pH 6.5). The size of the transcripts was estimated by comparison to molecular weight markers (RNA ladder; BRL). To ensure equal loading of RNA samples, a duplicate gel was run and stained with ethidium bromide to compare the intensity of the ribosomal bands. Gels were blotted onto GeneScreen (DuPont) membranes and hybridized with an 843-bp *X. laevis* AR PCR fragment (5) labeled by the random priming method to a specific activity of 1–5 × 10^9 cpm/μg. Hybridization was carried out in 50% formamide at 42°C for ≈16 hr. Blots were washed in 0.1× standard saline citrate at 62°C for 1 hr and exposed to x-ray film with an intensifying screen for 2 days.

Southern Blot Hybridization. Genomic DNA was isolated from the kidney of a female homozygous diploid (16) frog. Eight micrograms of DNA was digested with one of two restriction endonucleases, *Bam*HI or *Hind*III, overnight at

37°C, run on 0.9% agarose gels in 0.04 M Tris acetate/0.002 M EDTA buffer, and blotted onto GeneScreen (DuPont). A 500-bp EcoRI/EcoRI AR cDNA fragment, located within the eighth exon of the mammalian receptor (17), was labeled by the random priming reaction. Hybridization and washes were carried out under high-stringency conditions. The blot was exposed to x-ray film for 4 days.

[³H]Thymidine Autoradiography. We wished to compare the pattern of expression of AR mRNA isoforms to the pattern of androgen-evoked cell proliferation in the developing larynx. Proliferation of muscle and cartilage precursors is accelerated by treatment with exogenous androgens (8, 10). Thus, for this particular experiment, we exposed PM2 males to supraphysiological levels of 5α-dihydrotestosterone, a treatment that results in temporally compressed cell proliferation relative to the prolonged period (6–9 months; PM0–PM3) of cell proliferation in intact males. PM2 males were implanted with 5α-dihydrotestosterone-filled silastic tubing (0.25 mg per g of body weight) in the dorsal lymph sac. After 4 days, frogs were injected intraperitoneally with [³H]thymidine (1 μCi per g of body weight; 1 Ci = 37 GBq). Stage PM2 extends for ≈3 months of PM life (13); the duration of treatment in this study (4 days) was short relative to the duration of the stage and laryngeal weights after androgen treatment did not exceed the upper limit for PM2. Larynges were collected 24 hr after thymidine injection, fixed in 10% buffered formalin for 1–7 days, embedded in paraffin, sectioned transversely at 20 μm, and prepared for autoradiography as described (18). Slides were exposed for 21 days, developed in D-19 (Kodak), and counterstained with cresyl violet. The distribution of labeled cells was compared to the distribution of cells expressing AR mRNA isoforms.

In Situ Hybridization. Larynges were collected and fixed overnight in 4% paraformaldehyde at 4°C. Tissue samples were then dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Eight-micrometer sections were affixed to Superfrost plus (Fisher)-coated slides and stored at 4°C until hybridization. An 843-bp *X. laevis* AR cDNA fragment (5), or a 5′ 1.1-kb fragment (see Fig. 1), both constructed with RNA polymerase promoters at either end, were used as templates to generate antisense and sense transcripts by standard methods (19). To ensure that conditions of hybridization were comparable for the two probes,

equal counts (3 × 10⁶ cpm) of each were used per slide. Hybridization and washes were carried out under high-stringency conditions as described elsewhere (20, 21). Slides were dipped in Kodak NTB2 emulsion, exposed for 12 days, developed in D-19 (Kodak), and counterstained with cresyl violet.

RESULTS

Steroid receptor proteins contain discrete functional domains: a hormone binding domain located at the C terminus, a centrally located DNA binding domain, and a domain (the A/B or hypervariable domain) located at the N terminus. We wished to determine whether AR α and β mRNAs differ in regions encoding these domains. We approached this issue by screening a laryngeal cDNA library with the PCR-derived AR cDNA fragment (5). Newly isolated AR cDNA clones were hybridized to PM2 laryngeal cartilage RNA and scored for hybridization to one or another transcript. All clones that do not extend more 5′-ward than the DNA binding domain recognized both AR α mRNA (9.6 kb) and AR β mRNA (8.0 kb). Smaller PCR fragments generated from these clones encoding either the DNA binding or ligand binding domains alone also hybridized to both transcripts (Fig. 1). In contrast, a 1.1-kb cDNA fragment derived exclusively from the more 5′ A/B domain of one clone hybridized uniquely to the AR α mRNA (Fig. 1). We conclude that the AR α and β mRNAs differ within the hypervariable domain.

The AR α and β mRNAs could originate from one or more AR genes. As a step toward understanding the basis for expression of these two transcripts, we assessed AR gene copy number by genomic Southern blot hybridization (Fig. 2) with DNA isolated from a homozygous diploid animal since *X. laevis* is polyploid at many loci (16). The location of exon/intron boundaries of a given steroid hormone receptor is conserved across species and even across different steroid receptor genes (3); we expect that these boundaries will also be conserved for the *Xenopus* AR gene. A 500-bp EcoRI/EcoRI cDNA fragment from the *X. laevis* 3′ untranslated region (UTR), which would lie entirely within the large eighth exon (17) of the single mammalian AR gene (2–4), was thus used; this probe recognizes both the AR α and β transcripts in Northern analyses of developing *X. laevis* larynx (data not
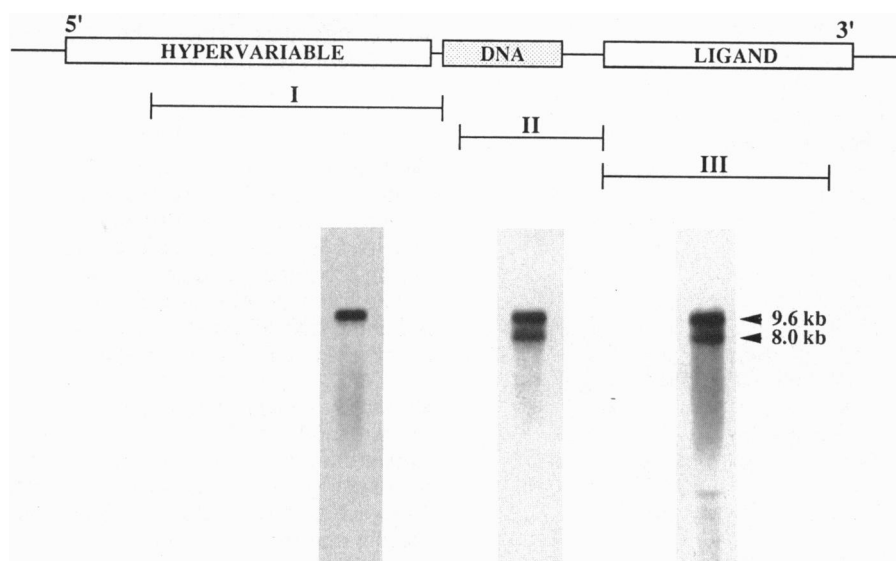

Fig. 1. Two AR mRNAs are present in developing larynx. (*Upper*) Schematic representation of the AR molecule. Positions of cDNA probes used in Northern analyses (*Lower*) are indicated. Probe I is a 1.1-kb cDNA fragment confined entirely to the 5′ hypervariable or A/B domain of the AR. Probes II and III are cDNA fragments from the DNA binding or ligand binding domains, respectively. Probe I hybridizes only to the AR α mRNA, while probes II and III hybridize to both the α and β transcripts.
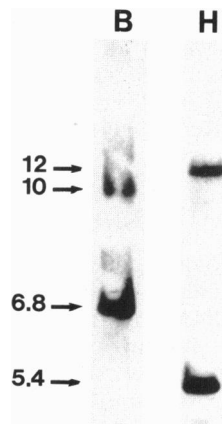
FIG. 2.   Southern analysis reveals at least two genes for the *X. laevis* AR. Genomic DNA was digested with *Bam*HI (lane B) or *Hind*III (lane H) and hybridized to a 500-bp *Eco*RI/*Eco*RI fragment from the 3′ UTR. Band sizes are indicated in kb.

shown). When the DNA was digested with *Bam*HI (B) or *Hind*III (H), neither of which cut the 500-bp sequence, two hybridizing fragments were detected (Fig. 2). We conclude that *X. laevis* has at least two AR genes. We do not yet know whether the AR α and β mRNAs originate from these two AR genes or are instead derived from a single gene.

We next explored the expression of AR α and β mRNAs during laryngeal masculinization. At early PM stages (PM1, not shown, and PM2), the larynx exhibits high levels of both AR mRNAs in laryngeal muscle and cartilage (Fig. 3). Both total AR mRNA levels and the β/α ratio are greatest in PM2 cartilage. As development proceeds (PM3), levels of both transcripts decrease. In adulthood (PM6), AR α mRNA is expressed within muscle but not in cartilage; AR β mRNA is undetectable. Because its expression is temporally restricted to early PM stages, we conclude that AR β is a developmentally regulated AR mRNA isoform. The results of Northern analyses suggested that AR β mRNA expression is associated with a specific phase of laryngeal masculinization, the androgen-evoked cell proliferation that occurs between PM0 and PM2, and that the later androgen-induced differentiation

of specific muscle or cartilage types that occurs between PM2 and PM5 (8–10) requires only expression of AR α mRNA.

We next wished to determine whether AR β mRNA is expressed specifically in the proliferative zones of the larynx that will give rise to specialized cartilages and muscle. Premature exposure to high levels of androgen induces marked cell proliferation in perichondrial and precartilage zones that will give rise to cells of the laryngeal cartilages and in myoblasts that will proliferate and then fuse to form laryngeal muscle fibers; cell addition in intact males is also dependent on androgen secretion but proliferation occurs over 6–9 months in juvenile males (8). We therefore exposed males to supraphysiological levels of androgen in order to induce rapid and extensive cell proliferation, which would facilitate spatial comparison with expression of AR mRNA isoforms. The androgen-stimulated PM2 male larynx contains large numbers of proliferating cells in the precartilage zone that will differentiate into specialized elastic cartilage (Fig. 4A). This zone was included in the cartilage component of Northern analyses of untreated males (Fig. 3). Cell division is also prominent within the hyaline cartilage, due to appositional growth from the perichondrium, and in developing muscle. Label in developing muscle is due to proliferation of myoblasts (ref. 8; these cannot be distinguished at the cellular level by light microscropy).

To localize AR α and β mRNAs, two AR probes were hybridized *in situ* to sections of unstimulated male PM2 larynx: the 843-bp cDNA fragment that recognizes both AR α and β mRNAs and the 1.1-kb cDNA fragment described above that recognizes only the α transcript. α/β hybridization reveals a striking concentration of AR mRNA in proliferative tissues, especially the elastic precartilage zone and the hyaline cartilage perichondrium (Fig. 4B). Hybridization with the α probe (Fig. 4C) reveals markedly less expression within these tissues than with the α/β probe. The extent of hybridization to differentiated hyaline cartilage, however, is equivalent for the α/β and α probes (compare Fig. 4 B to C), suggesting that this tissue, which is largely postmitotic at PM2, expresses only the AR α mRNA. In PM2 male muscle, labeling with the α probe is also less marked than with the α/β probe, suggesting that myoblast proliferation, which comes to an end by PM3, may also be associated with coexpression of the α and β mRNA isoforms. Thus, we conclude that both AR α and β mRNAs are expressed within proliferating tissues of the larynx; the β mRNA isoform is specifically associated with proliferating tissues, while the α mRNA isoform persists in differentiated tissues.

DISCUSSION

We had demonstrated previously that the developing larynx of male *X. laevis* expresses two AR mRNAs (5). Results presented here suggest that one of these AR mRNAs, AR β mRNA, represents an isoform that is transiently expressed during proliferative stages of masculinization. AR β mRNA is therefore a developmentally regulated AR mRNA isoform. In human LNCaP prostatic carcinoma cells, two AR mRNAs are present as the result of alternative splicing within the 3′ UTR (17). In rat brain, two AR mRNAs are derived from alternative splicing within the 5′ UTR (22). Thus, in mammals the AR mRNAs identified to date are predicted to encode similar or identical proteins; expression may be differentially regulated by the UTRs. The two AR mRNAs expressed in the *Xenopus* larynx, on the other hand, differ within the coding domain and presumably give rise to different proteins. The mammalian AR appears to derive from a single gene (2–4), while at least two AR genes exist in *X. laevis*. A more extensive molecular analysis is required to determine the relation of AR α and β mRNAs to the two AR genes of *X. laevis*.
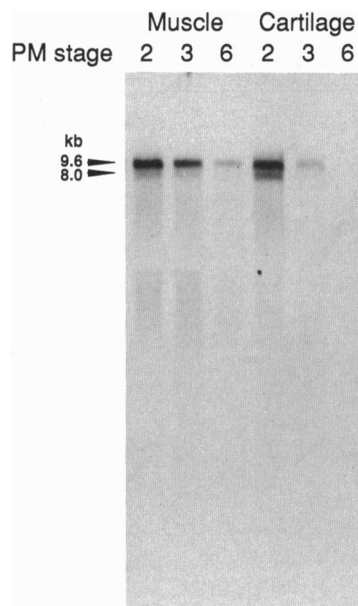

FIG. 3.   AR mRNAs are developmentally regulated. AR expression in male laryngeal muscle and cartilage at PM2, -3, and -6 was examined by Northern blot analysis.
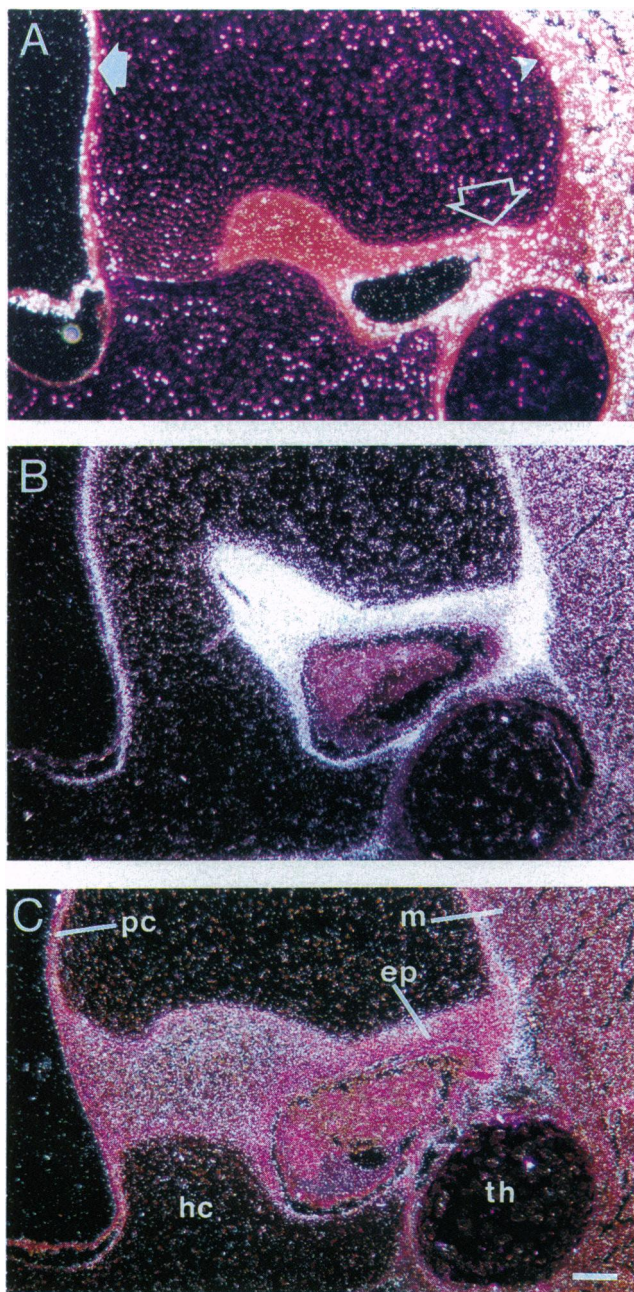
FIG. 4. Androgen-evoked cell proliferation and expression of AR mRNA isoforms in developing larynx. (A) Cell proliferation in developing larynx evoked by dihydrotestosterone and visualized by [³H]thymidine autoradiography. Proliferation is prominent in the elastic cartilage precursor zone (open arrow; this zone surrounds an unlabeled blood vessel), the developing muscle (arrowhead), and adjacent to the hyaline cartilage perichondrium (solid arrow). Hyaline and thyrohyal cartilages are, for the most part, postmitotic in PM2 males but proliferation can be induced with exogenous androgen. (B) *In situ* hybridization, using a probe that recognizes both AR α and β mRNAs, to a similar section through the larynx of a PM2 male. (C) *In situ* hybridization using a probe that recognizes only the AR α mRNA; section adjacent to that shown in B. ep, Elastic cartilage precursor zone; pc, perichondrium; m, muscle; hc, hyaline cartilage; th, thyrohyal cartilage. Sections adjacent to those shown in B and C hybridized with AR sense transcripts as a control for nonspecific hybridization revealed only a low level of nonspecific hybridization (data not shown). (Bar = 100 μm.)

AR β mRNA is expressed in areas of the larynx that are proliferating, while AR α mRNA is also expressed in differentiated tissues. If the AR β mRNA is associated with androgen-evoked cell proliferation, its expression should decrease as the larynx matures and proliferation ceases. Northern and *in situ* analyses support this expectation; expression of AR β mRNA diminishes as laryngeal cartilages stop dividing (8) (see Fig. 3). Similarly, in laryngeal muscle, expression of AR β mRNA is greater at PM2, when myoblasts are still proliferating (8), than at PM6, when myogenesis is complete (9). Thus, coexpression of proteins derived from the α and β mRNA isoforms in proliferating cells of the juvenile larynx could be causally related to androgen-evoked cell proliferation.

The two AR mRNA isoforms we have identified differ within the 5' hypervariable domain, a coding region. More subtle but as of yet undetected differences may also exist in the DNA and ligand binding domains. Of the different functional domains of the steroid receptors, the A/B domain is the most highly divergent (hence, hypervariable). This domain has been implicated in transcriptional activation, nuclear translocation, receptor dimerization, and target gene specificity (23). The β transcript of the *X. laevis* AR may lack an A/B domain entirely or it may contain a divergent A/B domain, which is not recognized by the α-specific probe.

Findings for other members of the steroid hormone receptor superfamily suggest several possible models for the functional consequences of A/B domain differences in the *X. laevis* AR. First, divergent A/B domains could confer differences in the activation of specific androgen target genes. For example, gene products critical for induction of cell proliferation may be activated specifically or more strongly by AR β. This mechanism appears to operate in the chicken progesterone receptor, which exists as two protein isoforms, A and B; A has a truncated hypervariable domain and can transactivate a specific reporter gene *in vitro*, which the full length form, B, cannot (24). Second, AR α and β may form heterodimers required to activate the genes underlying cell proliferation. For retinoic acid receptors, heterodimerization of one receptor (retinoid X receptor) with another (retinoic acid receptor) greatly increases the affinity of binding to DNA *in vitro* (25). In both of these models, expression of AR β increases the transcriptional activation of androgen target genes related to control of cell proliferation. Third, AR β may block AR α-induced cell differentiation and permit the continued proliferation of cartilage and muscle precursors. AR β could even be transcriptionally inactive (due, perhaps, to a missing A/B domain) but regulate the transcriptional activity of the α receptor via competition for available androgen or DNA binding sites. mRNA variants of unknown function for both thyroid and retinoic acid receptors lacking portions of the A/B domain have been described (26, 27). For the thyroid hormone receptor, an alternatively spliced variant, α2, lacking the ligand binding domain, inhibits gene expression normally activated by the α1 form (28). AR β would then function to maintain the plasticity of the developing male larynx through the prevention of premature differentiation.

Hormone-evoked cell proliferation is a prominent feature of sexual differentiation in many vertebrates and in some tumors of reproductive organs. The developing larynx of *X. laevis* relies absolutely on androgen secretion for the masculine pattern of cell addition and thus should prove a useful cellular model for understanding hormone-induced hyperplasia of other tissues, including cancers of the prostate, uterus, and breast.

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