Androgen Regulation of a Laryngeal-Specific Myosin Heavy Chain mRNA Isoform Whose Expression Is Sexually Differentiated

Diana S. Catz, Leslie M. Fischer, and Darcy B. Kelley
Department of Biological Sciences, Sherman Fairchild Center for the Life Sciences, Columbia University, New York, New York 10027

The larynx, the vocal organ of Xenopus laevis, is sexually differentiated; male laryngeal muscle fibers are entirely fast twitch while female fibers are predominantly slow twitch. In adults, all male laryngeal muscle fibers express the mRNA for a laryngeal-specific myosin heavy chain (MHC), LM; female laryngeal muscle expresses LM in a subset of fast-twitch fibers. In juvenile females, LM expression is increased by exposure to exogenous androgen, suggesting that sexually differentiated expression of this laryngeal-specific MHC is regulated by exposure to male sex hormones. Here we examine hormonal regulation of LM expression in juvenile male and female frogs. Exposure to exogenous androgen increases LM expression in both sexes. In situ hybridization analyses of larynges reveal upregulation of LM expression within 8 hr of androgen treatment in males; upregulation is not evident until after 48 hr in females. The upregulation in juvenile females includes both an increase in the number of muscle fibers expressing LM and an increase in expression in fibers already expressing LM. In juvenile males, all fibers express LM from the end of metamorphosis on; levels of LM expression are increased in all fibers by androgen treatment. Androgen-induced increases in LM expression are prevented by treatment with cycloheximide and are thus dependent upon protein synthesis. Castration of juvenile males results in diminished LM expression relative to intact animals. We conclude that expression of LM is regulated by exposure to androgen and that this regulation accounts for the sexually differentiated phenotype.

INTRODUCTION

The process of sexual selection (Darwin, 1871) has led to the evolution of physical features and behaviors that differ between the sexes and are associated with competition for mates. Many of these characteristics are found only in one sex, generally the male, because their expression requires the secretion of sex-specific hormones. The cellular and molecular mechanisms that underlie the expression of sexually differentiated characteristics are poorly understood. To approach this problem, we are examining a male-specific behavior, the courtship song of the clawed frog Xenopus laevis (see review see Kelley and Tobias, 1989) and its effector organ, the larynx. Male and female larynges have dimorphic features that allow only the male to sing (Ridewood, 1985; Wetzel and Kelley, 1983). To produce the rapid trills of male courtship song, laryngeal muscle must contract at 70 Hz (Wetzel and Kelley, 1983) while the female-typical release call is much slower, 7 Hz (Weintraub et al., 1985). The demands of sex-specific vocalizations are matched by sex differences in laryngeal muscle fibers: laryngeal muscle of adult males is entirely fast twitch while that of females is predominantly slow twitch (Sassoon et al., 1987).

Sex differences in laryngeal differentiation arise during postmetamorphic development and are regulated by androgenic steroids (Sassoon and Kelley, 1986, Sassoon et al., 1987, Tobias et al., 1991a,b). During early postmetamorphic development larynges of both sexes contain a mix of slow- and fast-twitch fibers, in response to testosterone androgen secretion in males, slow-twitch fibers convert to fast-twitch fibers (Sassoon et al., 1987, Tobias et al., 1991a,b). We isolated a partial cDNA clone for a myosin heavy chain (MHC)
isoform that is expressed preferentially in male laryngeal muscle (LM; Catz et al., 1992). During postmetamorphic development LM expression increases in male and declines in female larynges. Maximal expression of LM precedes laryngeal fiber type transformation (Catz et al., 1992). Three weeks of androgen treatment of juvenile females can induce fiber type transformation from slow to fast (Sassoon et al., 1987). Similarly, LM expression in juvenile females increases to male levels after 3 weeks of androgen treatment (Catz et al., 1992).

It is still not clear whether androgen exposure per se is the only functionally relevant factor in the sexual differentiation of laryngeal myosin isoform expression. The expression of many genes is androgen-dependent; in only a few, however, has it been demonstrated that transcription is directly regulated by androgens (Riehman et al., 1991). While androgens are known to promote muscle development and hypertrophy as well as myosin heavy chain isoform switching in target muscles (Lyons et al., 1986), little is known about how androgen regulates specific myosin gene expression. The aims of this study were to investigate the time course of androgen regulation of LM mRNA expression in juvenile male and female laryngeal muscle, the dependence of androgen-induced LM mRNA upregulation on protein synthesis, and the effect of castration on LM mRNA expression in juvenile males.

MATERIALS AND METHODS

Animals

*Xenopus laevis* frogs were obtained from Nasco (Ft. Atkinson, WI). Juveniles were studied at two stages: PM0, shortly after the completion of metamorphosis, when frogs weigh 1–2 g, and PM2, when frogs are ~6 months old and weigh 5–7 g (stages of Tobias et al., 1991a). At PM0, when larynges weigh 3–10 mg, laryngeal muscle fibers are primarily slow twitch in both sexes, masculinization of fiber type begins at PM2. Characteristic male laryngeal weights for PM2 are 36–70 mg (Tobias et al., 1991a).

Hormone Treatments

Dihydrotestosterone propionate (DHT, Sigma), 0.25 mg/g body wt, was administered to each PM2 frog in Silastic tube implants (Dow Corning, Midland, MI) procedure as in Watson and Kelley (1992). When juveniles are treated for 4–5 weeks with this dose of androgen, all muscle fibers switch to fast twitch (Tobias et al., 1991b, 1993). Hormone implants were placed into the dorsal lymph sacs of the frogs and positioned immediately posterior to the orbits. At 0, 4, 8, 48, 96, and 240 hr after implantation, frogs were anesthetized by immersion in buffered 0.1% MS-222 (m-aminobenzoate, methanesulfonic acid, Sigma), sexed by gonadal inspection, and killed. At each time point, 25 frogs (mixed sex groups of males and females) were used and the entire experiment was repeated three times. At each time point, 4 male and 4 female larynges were prepared for *in situ* hybridization; the remaining larynges were used for Northern analyses.

Cycloheximide Treatment

PM2 males were injected intraperitoneally with saline (0.65% NaCl, 100 µL, N = 6) or cycloheximide (0.65 mg in 100 µL saline, N = 6) and implanted 2 hr later with DHT as described above. This dose of cycloheximide was chosen because it has been shown to block protein synthesis in adult *Xenopus laevis* (Ayoob et al., 1992). The animals were injected again with saline or cycloheximide 6 hr later and sacrificed 8 hr after DHT implantation. Untreated males (N = 6) served as controls. Larynges were prepared for *in situ* hybridization as described below.

Hormone Treatments—Blood Levels of Androgen

Total androgen levels were measured in all short- and long-term androgen-treated PM2 animals and in a separate group of frogs 2, 4, and 6 hr after hormone implantation. At the time of sacrifice, blood was collected from individual animals by cardiac puncture and then spun at 12,000 rpm to remove red blood cells. Pooled serum samples of androgen-treated animals were subjected to radioimmunoassay (Amersham) following standard procedures.

Gonadectomy

PM0 and PM2 frogs were anaesthetized by immersion in 0.1% MS 222 (ethyl m-amino benzoate methane sulfonic acid, Aldrich Chemical Co.) and castrated as described previously (Marin et al., 1990). Castrated animals were reared for 3 weeks and then killed. Age-matched, sham-operated juveniles killed at the same time served as controls.

Northern Blotting

Total RNA was prepared by the RNAzol method (TEL-TEST, Inc.). RNA was denatured using glyoxal (McMaster and Carmichael, 1977). 10 µg of total RNA was loaded in each lane and electroeluted separately on 1.2% agarose gels. Gels were blotted onto GeneScreen membranes (DuPont) by standard methods. Hybridization and washes were carried out under high stringency conditions using a 32P-labeled *PstI/EcoRI* fragment of clone F3 (F3-500) as described previously (Catz et al., 1992). This cDNA fragment (EMBL/GenBank Accession No. L01495) consists of 312 nucleotides coding for 104 C-terminal amino acids and 144 nucleotides from the 3’ noncoding region; highest homologies at the amino acid level are to other sequenced vertebrate MHC's. Ethidium bromide staining of parallel gels or methylene blue staining of membranes was used to control for amount loaded on the gels. After F3-500 hybridization, some membranes were stripped and reprobed sequentially.
with elongation factor EF1α [Krieg et al., 1989] and then an embryonic muscle actin (clone H2 of Dworkin-Rassel et al., 1986; Mohun et al., 1988). Under these conditions, the H2 actin probe hybridizes to three bands in Northern analyses of thigh muscle from PM2 females (not shown) but to only a single band, corresponding to the middle band of thigh muscle, in Northern of PM2 laryngeal muscle.

Densitometric analysis of Northern blot autoradiograms hybridized with the LM, H2, or EF1α probes was performed using a Bioimage video densitometry system (Millipore) as described previously [Crothers et al., 1993]. Integrated optical densities of bands corresponding to LM, H2, and EF1α were obtained for time points 0, 4, 8, 48, 96, and 240 hr after DHT treatment. To control for gel loading, values for LM and H2 expression were divided by the EF1α expression value for that lane relative to the 0 hr female time point.

**In Situ Hybridization**

Male and female larynges were processed for *in situ* hybridization as described in Catz et al., 1992. Briefly, tissue was collected, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated, and embedded in paraffin. Ten-micrometer transverse sections from comparable regions of the larynx were deparaffinized, treated with proteinase K, incubated in 0.2 N HCl, and then treated with proteinase. Antisense or sense RNA probes were transcribed using T7 or T3 RNA polymerase (Promega Biotech), respectively. The RNA probe used for *in situ* hybridization was synthesized from a 0.5-kb EcoRI/NotI fragment of the F3 clone subcloned into the polylinker of pBluescript [Stratagene]. We used linearized F3-500 plasmid in 20-μl reaction mixtures containing 50 μCi [35]UTP (Catz et al., 1992). Typically each slide was hybridized with 3 × 10^6 cpm of probe overnight at 50°C. The next day slides were washed in 2× SSPE, treated with RNase, and washed again in 50% formamide, 2× SSPE at 60°C. Washed slides were dipped into Kodak NTB-2 emulsion and exposed for 7 days. Under these hybridization conditions in adult mouse, above background labeling is present only in the dilator laryngis, no expression of LM is detected in an extrinsic laryngeal muscle (*genioglossus internus*), another androgen-regulated muscle (*sternocleidomastoideus*), another fast muscle (*tibialis anterior*), or in cardiac muscle [Catz et al., 1992]. After development in D-19, sections were counterstained with cresyl violet. For all sections hybridized with sense RNA probes, labeling did not exceed background.

**RESULTS**

**Androgen Treatment Increases LM Expression in PM2 Females**

Within 2 hr of DHT implantation, supra-physiological levels (>10 ng/ml) were detected in serum, levels peaked by 4 hr (14 ng/ml) and were maintained at 6 hr. Supra-physiological levels (>10 ng/ml) were present at all subsequent time points.

Northern analyses reveal, as expected (Catz et al., 1992), sexually differentiated levels of LM expression in untreated PM2 larynges (Fig. 1A, 0 hr lanes). A quantitative analysis reveals that levels of LM expression in PM2 male laryngeal muscle are 10 times those in female laryngeal muscle (Fig. 1B, 0 hr). When females are treated for 10 days (240 hr) with androgen, LM expression is markedly increased (6× control values; Fig. 1B). In males, androgen does not have a similarly dramatic effect. The largest increase observed at 8 hr is only 1.24× the control value (Fig. 1B). In females but not in males, 8 hr of DHT treatment produces an apparent decrease in LM expression (Figs. 1A and 1B). The same results were obtained in two additional replications of the Northern analyses. The effects of androgen treatment can be compared to the effects on expression of an embryonic actin, H2. Unlike LM, there is no specific induction of H2 actin expression following androgen treatment in either sex (Figs. 1A and 1B).

We conclude that levels of LM mRNA expression in PM2 male larynges exceed those of females. Exogenous androgen increases LM mRNA expression markedly in juvenile female but not male larynges, the effect requires an extended period of treatment. In females, a transient decrease in LM mRNA expression appears shortly after exposure to DHT.

We next examined the pattern of LM mRNA expression in laryngeal muscle of juvenile frogs after androgen treatment using *in situ* hybridization. In untreated females at PM2, LM mRNA expression in laryngeal muscle appears patchy (Fig. 2, 0 hr female). No effects of DHT treatment on LM mRNA expression are apparent (not shown) or 8 hr after DHT treatment (Fig. 2, 8 hr). After 48 hr of DHT treatment, expression of LM in female laryngeal muscle is uniform and increased relative to previous time points (Fig. 2, 48 vs 8 hr; see also Fig. 3, female, 48 vs 8 hr for higher magnification). At 96 hr (Fig. 2) and 240 hr (Fig. 2, 20 day) after DHT treatment, LM mRNA expression is high and uniform and female laryngeal muscle is hypertrophied relative to untreated controls. In untreated males at PM2, LM mRNA expression is uniform (Figs. 2 and 3, 0 hr male).

The effects of DHT on LM expression in male larynges are illustrated in Fig. 3. Expression of LM in PM2 male laryngeal muscle is unchanged after 4 hr of DHT treatment (not shown) but after 8 hr of treatment LM expression is increased in every fiber (Fig. 3, 8 hr male; see also Fig. 4). Expression of LM in males continues to increase, reaching maximal levels after 96 hr of DHT treatment (not shown).

We conclude that a sexually dimorphic pattern of LM mRNA expression is present in laryngeal muscle of PM2 juveniles. In untreated males, expression is uniform and higher than in females, where expression is patchy. Treatment with exogenous androgen increases LM expression in both sexes; upregulation in males occurs more rapidly (by 8 hr) than in females (by 48 hr). The apparent decrease in LM mRNA expression seen in Northern blots in females 8
FIG. 1. Regulation of a laryngeal myosin heavy chain isoform (LM) expression by treatment with androgen. Gonadally intact female and male PM2 frogs were treated with dihydrotestosterone (DHT) for the times indicated in hours. (A) 10 μg of total RNA extracted from laryngeal muscle was loaded in each lane, electrophoretically separated, and subjected to Northern analysis using F3-500 as a probe. This blot was simultaneously probed with the elongation factor EF1α (Kriegl et al., 1989) and then stripped and reprobed with an embryonic actin (clone H2 of Dworkin-Rastl et al., 1986). Autoradiograms were exposed overnight. (B) Densitometric analysis of the autoradiograms shown in A. Integrated optical density values were obtained for each band on the autoradiogram. To control for gel loading, values for LM and H2 expression were divided by the EF1α expression value for that lane relative to the 0 hr female time point.

hr after DHT treatment (Figs. 1A and 1B) is not present in in situ autoradiograms.

De Novo Protein Synthesis Is Required for DHT-Induced Increases in LM Expression

We next determined, using in situ hybridization, if de novo protein synthesis is required for the upregulation of LM by androgen. We used only males for this experiment because of their more rapid response to androgen. By 8 hr after exposure to DHT, expression of LM was elevated in laryngeal muscle of PM2 males (Fig. 4, middle) relative to untreated controls (Fig. 4, left). When cycloheximide is administered prior to hormone treatment (Fig. 4, right), DHT-induced upregulation of LM expression does not occur. We conclude that cycloheximide, an inhibitor of protein synthesis, blocks androgen-induced increases in LM mRNA expression in PM2 male laryngeal muscle.

Effects of Castration on LM Expression

We wished to determine whether endogenous testicular androgen secretion in males is responsible for sex differences in LM expression in juveniles. Thus, we examined, using in situ hybridization, LM expression in larynges of castrated PM0 and PM2 frogs as compared to intact con-

FIG. 2. In situ hybridization analysis of androgen-regulated LM expression. Dark-field micrographs of transverse 10-μm hemisections through the larynges of female PM2 frogs treated with DHT for 0, 8, 48, 96, or 240 hr (240 hr = 10 days) are shown. For comparison, hybridization of an antisense LM probe to an untreated PM2 male larynx (panel at lower right, 0 hr) is also shown. The hybridization of the LM probe to an extrinsic muscle of the larynx, the strap muscle, did not exceed background (see arrowheads, male 0 hr), control hybridization with sense probes also did not exceed background (not shown). In the section of female larynx at 0 hr, a fold immediately above the thyrohyal cartilages (TH) gives a false impression of increased expression relative to the rest of the muscle. LM expression is patchy throughout the muscle. Scale bar, 250 μm.
FIG. 3. Higher magnification in situ hybridization autoradiograms of LM expression in male and female PM2 larynges after androgen treatment, dark-field illumination. In females, LM expression after 8 hr of DHT treatment (top left) is patchy and does not exceed control values (see Fig. 2). After 48 hr of treatment, expression of LM in female larynges is uniform and increased relative to previous time points (bottom left). In PM2 males, expression of LM is uniform before treatment (top right) and increased after 8 hr of DHT treatment (bottom right). Scale bars, 100 μm.
trols (Fig. 5). As was observed previously [Catz et al., 1992], the level of expression of LM mRNA in male laryngeal muscle is higher at PM2 than at PM0 (Fig. 5, compare top panels). Castration reduced LM expression, relative to intact controls, at both PM0 and PM2 (Fig. 5, compare top to bottom). The level of LM expression in castrated PM0 males did not exceed background. We conclude that the male-specific increase in LM expression during early juvenile development is dependent on the presence of the testes.

DISCUSSION

In *Xenopus laevis*, laryngeal muscle of the adult male is entirely fast twitch while that of the female is largely slow twitch [Sassoon et al., 1987]. This sex difference in vocal muscles underlies the production of rapid trills by males and slow trills by females [Tobias and Kelley, 1987; Tobias et al., 1991a,b]. Sexual differentiation of muscle fiber type relies on testicular androgen secretion [Sassoon et al., 1987; Tobias et al., 1991a,b; Watson and Kelley, 1992]. While the advantages of androgen-directed differentiation of laryngeal muscle fiber type for sexually selected vocal behaviors are evident, it was not clear how this particular characteristic is conferred upon vocal muscles. The cloning of a laryngealspecific myosin heavy chain isoform, LM, whose expression could be increased by exogenous androgen [Catz et al., 1992], suggested that the underlying mechanism could involve hormonal regulation of highly tissue-specific muscle proteins. In adult males, LM is expressed in all muscle fibers and all fibers are fast twitch [Catz et al., 1992; Sassoon et al., 1987]. In adult females, LM is expressed in a subset of fast-twitch fibers. Based on these results, we have hypothesized that sexually differentiated LM expression is responsible for the sex differences in twitch properties of laryngeal muscle fibers.

Sexual differentiation of LM expression precedes sexual differentiation of muscle fiber type. At PM1, LM is already more highly expressed in males than in females; LM expression in females peaks at PM2 and then declines [Catz et al., 1992]. LM expression in postmetamorphic animals may reflect the degree of androgen exposure and sex differences arise in consequence. The present results provide evidence for this hypothesis. Ablation of the testes decreased LM expression in males and provision of exogenous androgen increased LM expression in females. We could not detect above background levels of LM expression in males castrated at PM0. This result suggests that without some androgen secretion LM may not be transcribed in the larynx. Both juvenile and adult females secrete some androgen [Kang et al., 1994], which could account for the persistence of LM expression in female larynges [Catz et al., 1992]. If this hypothesis is correct, antiandrogen treatment at early PM stages should prevent LM expression in females as it does in males.

How rapidly does LM expression follow exposure to androgen? The results of Northern and in situ hybridization analyses give conflicting answers to this question. In the in situ autoradiograms, DHT-induced increases in LM expression are apparent after 8 hr in males and after 48 hr in
Androgen Regulation of Myosin

females. In Northern, there is only a slight increase at the 8-hr time point in males and no increase at 48 hr in females. In fact, DHT treatment induces an apparent decrease in LM expression at the 8-hr time point in females. A similar discrepancy between the results of Northern and in situ hybridization analyses is also seen for androgen receptor mRNA expression in Xenopus laevis (Fischer, 1983, Fischer et al., 1995) and for androgen receptor mRNA in the rat prostate (Quanby et al., 1990 vs Takeda et al., 1991). The most likely explanation for the discrepancy is that androgen is a very powerful promoter of growth in both the prostate and the larynx. In our Northern analyses, approximately equal aliquots of total RNA were loaded onto each lane of the gel. Under these conditions, the extent of hybridization of a specific probe to an RNA species will strongly reflect the abundance of the total number and amount of RNA species in the aliquot. If other members of the total RNA pool (e.g., ribosomal RNAs) are increased out of proportion to the DHT-induced increase in LM, the expression of LM will appear to decrease. In contrast, in situ analyses are not influenced by the presence of other transcripts in the cell and are thus more accurate and more sensitive indicators of androgen effects on LM expression.

Northern analyses are useful for comparing basal levels of expression in the sexes; our observations suggest that the expression of LM in male PM2 larvae is 10× female levels. The level of expression was quantified using optical density measurements and small differences in loading were compensated for by comparison to a ‘housekeeping’ gene, EF1α. This peptide elongation factor (now known as EF1α, Dje et al., 1990) is expressed in all embryonic cells of Xenopus laevis after the mid-blastula transition; it is associated with polysomes and its accumulation in gastrula is presumed to be involved in later demands for rapid protein synthesis (Krieg et al., 1989). Given the very powerful effects of androgen on growth of the juvenile larynx (Sassoon et al., 1986; Marín et al., 1990; Fischer et al., 1995), it is possible that EF1α expression was also affected. If so, this correction procedure could lead to an underestimate of androgen-induced changes in LM expression in both sexes.

The embryonic actin probe we used (H2) was also isolated from a gastrula library and is expressed strongly in developing muscles of all types (Dworkin-Rastl et al., 1986). Unlike LM, H2 actin expression is not androgen-induced. We do not know if H2 actin expression contributes significantly to laryngeal actin proteins. Because it is not coordinately regulated with LM, H2 actin is unlikely to be LM’s partner.

Most androgen-sensitive nonreproductive organs, like the kidney, undergo only hypertrophic responses to androgen administration. The mammalian male reproductive tract and the Xenopus laevis larynx, however, respond to androgen with both hyperplasia and hypertrophy (reviewed in Kelley, 1995, and Berger and Watson, 1989, respectively). Androgen-regulated genes in nonreproductive organs such as kidney are induced at a relatively slow rate. Maximal activation of mRNA synthesis of these genes requires between 2 and 10 days (Watson and Paigen, 1990). Androgen-responsive genes from the mammalian reproductive tract display more rapid activation (Felder et al., 1988). For example, prostate-specific antigen mRNA expression is increased within 6 hr after addition of androgens and reaches its maximum at 24 hr (Rieger et al., 1991). The mRNA for PBP-C3 increases after 2 hr of hormone treatment in castrated rats, while the mRNA for the subunits C1 and C2 are detectable after 8 hr of androgen treatment (Zhang and Parker, 1985). We show here that the laryngeal myosin heavy chain isoform LM is induced at maximal levels after 8 hr of DHT treatment in males and after 48 hr in females. In its relatively rapid response to androgen, LM resembles hormone-regulated genes of the mammalian male reproductive tract.

Another way of distinguishing classes of hormone-evoked gene responses during development is to ask whether they require de novo protein synthesis, i.e., are cycloheximide-sensitive (Buckbinder and Brown, 1992). We show here that prior treatment with cycloheximide blocks androgen-induced increases in LM expression. A further distinguishing feature of hormone-regulated genes is the time course of regulation: some responses are immediate and some delayed, some responses are transient while others are long lasting or “stable” (Horne and Heit, 1994). The regulation of LM suggests that it belongs to the delayed (hours) and stable category of this taxonomy. Like other genes in this category (e.g., globin) expression of LM is highly tissue-specific and hormone-regulated expression may play an important role in the creation and maintenance of a differentiated cell state. However, it is worth pointing out that changes in gene expression in response to thyroid or steroid hormones, even cycloheximide-resistant responses, are often slower than responses to other hormones. For example, the upregulation of the thyroxine receptor β gene by thyroid hormone is one of the earliest, cycloheximide-resistant responses to hormone stimulation but is not seen until between 4 and 8 hr after hormone exposure (Wang and Brown, 1993).

What characteristics of laryngeal muscle permit regulation of a myosin heavy chain gene by androgenic steroids? The developing larynx expresses extraordinarily high levels of androgen receptor (Kelley et al., 1989, Fischer et al., 1993) and it is likely that androgen acts to regulate LM expression in juveniles through activation of its receptor. What the requirement for protein synthesis implies about possible receptor-mediated mechanisms underlying LM regulation is not clear. A common assumption in the field is that cycloheximide-resistant ‘early’ gene responses are due to occupancy of a cis-acting hormone response element (HRE) for the gene in question; simple occupancy and binding to the HRE would thus be sufficient for activation or repression. However, even the best characterized androgen-regulated gene (Claessens et al., 1989, De Vos et al., 1991)—prostatic binding protein (PBP-C3)—requires protein synthesis for mRNA stimulation even though it has a functional intronic androgen response element. The blockade of androgen-induced increases in LM expression by cycloheximide thus does not rule out the possibility that the LM gene is regu-
FIG. 5. Castration reduces LM expression in juvenile frogs. Hybridization of the antisense LM probe to intact (top) or castrated (bottom) PM2 [left] or PM0 [right] is shown. Note that only a small portion of laryngeal muscle is depicted for the PM2 intact male; the region of muscle shown is similar to the "tongue" of muscle wrapping around the cartilage at bottom right in Fig. 2. The bright spots surrounding the laryngeal muscle are pigment granules. Scale bar, 100 μm.

related via an androgen response element. The cycloheximide effect may reflect a requirement for certain proteins with very short half lives present in limiting amounts or for some other receptor accessory factor (Kupfer et al., 1993) or transcription factor.

In conclusion, we have demonstrated the regulation of a tissue-specific myosin heavy chain gene by androgens. The secretion of testicular androgen appears responsible for the sexually differentiated levels of LM expression in adult muscle described previously (Catz et al., 1992). This gene is relevant for the masculinization of the frog larynx, and its regulation resembles patterns of regulation of genes of the male reproductive tract. The developmental and hormonal regulation of LM expression should provide insight into the poorly understood cellular and molecular cascade required for differentiation of the masculine phenotype.

ACKNOWLEDGMENTS

We thank members of the laboratory for useful comments on the manuscript. This work was supported by NIH Grant NS23584.

REFERENCES


developmental control encode elongation factor 1-a in *Xenopus laevis*. Nucleic Acids Res. 18, 3499–3499.


Krieg, P., Varmus, S., Wormington, W., and Melton, D. (1989). The mRNA encoding elongation factor 1-a (EF-1a) is a major transcript at the midblastula transition in *Xenopus*. *Dev. Biol.* 133, 93–100.


Received for publication January 31, 1995

Accepted June 14, 1995