Published in final edited form as:

Dev Biol. 1992 December ; 154(2): 366–376.

Sexually Dimorphic Expression of a Laryngeal-Specific, Androgen-Regulated Myosin Heavy Chain Gene during *Xenopus laevis* Development

Diana S. Catz, Leslie M. Fischer, Maria C. Moschella¹, Martha L. Tobias, and Darcy B. Kelley

Department of Biological Sciences, Sherman Fairchild Center for the Life Sciences, Columbia University, New York, New York 10027

Abstract

Masculinization of the larynx in *Xenopus laevis* frogs is essential for the performance of male courtship song. During postmetamorphic (PM) development, the initially female-like phenotype of laryngeal muscle (slow and fast twitch fibers) is converted to the masculine form (entirely fast twitch) under the influence of androgenic steroids. To explore the molecular basis of androgen-directed masculinization, we have isolated cDNA clones encoding portions of a new *Xenopus* myosin heavy chain (MHC) gene. We have detected expression of this gene only in laryngeal muscle and specifically in males. All adult male laryngeal muscle fibers express the laryngeal myosin (LM). Adult female laryngeal muscle expresses LM only in some fibers. Expression of LM during PM development was examined using Northern blots and *in situ* hybridization. Males express higher levels of LM than females throughout PM development and attain adult levels by PM3. In females, LM expression peaks transiently at PM2. Treatment of juvenile female frogs with the androgen dihydrotestosterone masculinizes LM expression. Thus, LM appears to be a male-specific, testosterone-regulated MHC isoform in *Xenopus laevis*. The LM gene will permit analysis of androgen-directed sexual differentiation in this highly sexually dimorphic tissue.

INTRODUCTION

In vertebrates, the development of sexually differentiated tissues is largely controlled by endocrine secretion from the gonads. The recent elucidation of molecular interactions between steroid hormones and target genes in a variety of tissues has greatly aided our understanding of hormone action (Evans, 1988). We still lack, however, an understanding of the molecular and cellular cascade, set into action by binding of hormone to its receptor, that ultimately results in the expression of male and female phenotypes. To address this issue we are examining how androgenic hormones regulate the development of the masculine phenotype in a sexually dimorphic tissue, the larynx of the clawed frog *Xenopus laevis*.

The larynx produces the vocalizations used by males and females during sex-specific courtship behaviors (Ridewood, 1898; Wetzel and Kelley, 1983; Hannigan and Kelley, 1986; Tobias and Kelley, 1987). Males produce rapid trills that attract females. Females produce slow trills that repel males. These vocalizations are produced by contraction of laryngeal muscles. Male laryngeal muscle contains entirely fast twitch/fatigue-resistant fibers while female laryngeal muscle contains both fast and slow twitch fibers (Sassoon *et*

al., 1987). Thus, sexual differences in vocal behavior are subserved by sex differences in laryngeal muscle properties. In *X. laevis*, as in other species, the contraction rate of individual fibers is controlled by the myosin heavy chain isoform expressed (Lannergren, 1987). We have thus predicted that sex differences in fiber type within the *X. laevis* larynx reflect sex differences in myosin heavy chain (MHC) expression. In this paper we identify a laryngeal-specific MHC gene which is expressed predominantly in males.

Sex differences in laryngeal fiber type are established during the first year following metamorphosis (Sassoon *et al.*, 1987; Tobias *et al.*, 1991a). At the end of metamorphosis, laryngeal muscle contains a heterogeneous population of slow and fast fibers in both sexes. Using ATP-ase histochemistry, we have determined when masculinization of fiber type occurs (Tobias *et al.*, 1991a). In males, the proportion of fast twitch fibers in the larynx begins to increase at postmetamorphic (PM) stage 2; by PM5, male laryngeal muscle is entirely fast twitch. Changes in fiber type are due to conversion of existing slow twitch to fast twitch fibers (Tobias *et al.*, 1991a, b). The female larynx maintains a heterogeneous muscle fiber population throughout postmetamorphic development. Laryngeal muscle conversion is regulated by androgens. Administration of androgen to juvenile male or female frogs rapidly switches the heterogeneous population of muscle fibers to all fast twitch. Castration blocks fiber type conversion in juvenile males (Sassoon *et al.*, 1987; Tobias *et al.*, 1991b). We thus examine whether expression of the sexually differentiated MHC identified here is affected by androgen and whether its expression follows the same temporal pattern as fiber type conversion.

MATERIALS AND METHODS

Animals

Xenopus laevis frogs at different stages of postmetamorphic development were obtained from Nasco (Ft. Atkinson, WI). Postmetamorphic stage was determined as described previously (Tobias *et al.*, 1991a). In a separate experiment, PM2 females and males were implanted with dihydrotestosterone propionate (DHT, Sigma) pellets (0.5 mg/g body weight) for 3 weeks (procedure in Tobias *et al.*, 1991b) and then were sacrificed.

Isolation of cDNA clones

For isolation of cDNA clones encoding *Xenopus* MHCs, we screened a cDNA library constructed in λ ZAP II (Stratagene) which had been prepared with a pool of poly(A)⁺ RNA from juvenile and adult, male and female larynges. As hybridization probes we used a gel-purified 320-bp *EcoRI/PstI* fragment of postmetamorphic skeletal muscle MHC clone X₁mbHC A₇, or a 1.4-kb *EcoRI* fragment of early swimming tadpole MHC clone XL myHC E₁₉ (Radice and Malacinski, 1989), kindly provided by Dr. Radice (Indiana University, Bloomington, IN). Hybridization was carried out under low and high stringency conditions (Maniatis *et al.*, 1982). Positives from both low and high stringency conditions were purified. The Bluescript plasmids containing the clones were *in vivo* excised from the λ vector following the manufacturer's recommended protocol.

Northern Blotting

Total RNA was prepared by the RNazol B method (TEL-TEST, Inc.). Purified RNA was denatured with glyoxal (McMaster and Carmichael, 1977), subjected to electrophoresis on 1.2% agarose gels, and blotted onto GeneScreen membranes (DuPont) following the manufacturer's protocol. Hybridization was overnight at 42°C and subsequent washes were at room temperature in 2×SSC, 0.1% SDS and at 65°C in 0.1×SSC, 0.1% SDS.

Sequencing

The F3, H11, and G12 clones were partially sequenced, and the f3-500 subclone was fully sequenced in both orientations with the Sequenase kit (U.S. Biochemicals), following the procedures recommended by the manufacturer for double-stranded DNA. Sequences obtained were compared to vertebrate MHCs from GenBank using the FASTA program.

In Situ Hybridization

The RNA probe used for *in situ* hybridization was synthesized from a 0.5-kb *EcoRI/PstI* fragment of the F3 clone subcloned into the polylinker of pBluescript (Stratagene) or from the entire 1.2-kb H11 clone by standard methods. Antisense and sense RNA were transcribed from linearized plasmids in 20 μ l reaction mixtures by T7 or T3 RNA polymerases (Promega Biotech), respectively, in the presence of 50 μ Ci [35 S]UTP or 3.5 mM of digoxigenin-11-UTP. Male and female larynges at representative PM stages (Tobias *et al.*, 1991a) were processed for *in situ* hybridization using a modification of existing protocols (Cox *et al.*, 1984; Kintner and Melton, 1987; Toran-Allerand and Miranda, 1992) and high stringency conditions. Paraffin-embedded, transversely sectioned material (10 μ m) was used for radioactive probes, and cryostat-sectioned material (30 μ m) was used for the digoxigenin-labeled probes. Typically, 100 μ l of hybridization solution containing 3×10^6 cpm of radioactive or 1.25 μ g of nonradioactive probe was used per slide. After the high stringency washes, slides hybridized with nonradioactive probe were blocked for 30 min at room temperature with 5% nonfat dry milk (Carnation) in Tris-buffered saline (TBS, 10 mM Tris, 140 mM NaCl, pH 7.4) containing 0.3% Triton X-100 and then were incubated for 48 hr at 4°C in a 1:1600 dilution of the polyclonal sheep anti-digoxigenin antibody Fab fragment conjugated to alkaline phosphatase (Boehringer-Mannheim), prepared in the blocking solution. Binding of the alkaline phosphatase-labeled antibody was visualized with a color reaction (3 hr at room temperature), using nitroblue tetrazolium salt (45 μ l) and 5-bromo-4-chloro-3-indolyl phosphate (35 μ l) as substrates (Genius kit, Boehringer-Mannheim). Slides hybridized with radioactive probe were dipped in NTB2 nuclear emulsion (Eastman Kodak Co.), exposed for 3–5 days, and were developed in D-19.

Combined in Situ Hybridization and Immunohistochemistry

The sections were treated as in the standard *in situ* hybridization procedure, using a lower concentration (1.5 μ g/ml) of proteinase K and incubating for only 15 min. After the washes, the slides were treated with PBS and prepared for immunohistochemical staining as recommended in the Vectastain ABC kit (Vector Laboratories). We used a monoclonal antibody (IC9) that recognizes a MHC from the tibialis anterior muscle of *Rana pipiens* (kindly provided by Dr. Martin Jones, University of California, San Francisco, CA). After the immunostaining, the slides were dipped in the emulsion as previously and exposed for 5 days.

RESULTS

Isolation and Characterization of Male-Specific MHC cDNA Clones

Our goal was to isolate specific myosin isoforms which are preferentially expressed in male laryngeal muscle. Using two cDNA myosin probes from *Xenopus* (see Materials and Methods), several clones were isolated from a laryngeal cDNA library. Because 3' regions of MHCs (particularly noncoding regions) are less conserved between different MHCs than other regions and because there is a 3' *PstI* site in several different clones of *X. laevis* MHCs (Radice and Malacinski, 1989), *PstI/EcoRI* fragments of our clones served as gene-specific probes in Northern blots containing male and female total laryngeal RNA. With this strategy we identified a clone, F3, that recognizes a 6.2-kb mRNA more abundant in male

than in female adult laryngeal muscle (Fig. 1A). The same blot was reprobed with a cardiac actin probe (Dworkin-Rastl *et al.*, 1986) to verify that each lane contained similar amounts of RNA (Fig. 1B). Two other clones were also male specific by this assay: G12 and H11 (data not shown). Partial sequencing of the three clones revealed overlapping sequences at their ends. H11 is the most 5' clone and contains only coding regions. All three clones exhibited about 70% nucleotide homology to other sequenced vertebrate MHCs (data not shown).

Figure 2 shows the DNA and predicted amino acid sequence of the *EcoRI/PstI* fragment of clone F3 (f3-500), used as a laryngeal myosin (LM) probe. This cDNA fragment consists of 312 nucleotides coding for 104 C-terminal amino acids and 144 nucleotides from the 3' noncoding region. The highest homology found at the amino acid level was to rat β cardiac MHC (79%; McNally *et al.*, 1989), to human embryonic MHC (79%; Stedman *et al.*, 1990), and to chicken embryonic MHC (78%; Molina *et al.*, 1987).

Expression of LM in Adult *Xenopus laevis*

We next wished to determine if the lower level of LM expression observed in females is associated with a particular type of fiber or if it is due to expression of LM in fewer fibers. The expression of LM in individual fibers of laryngeal muscle from adult male and female frogs was examined using a digoxigenin probe conjugated to alkaline phosphatase in the *in situ* hybridization procedure (Fig. 3). The expression of LM was confined to laryngeal muscle and no expression was detected in laryngeal cartilage or connective tissue (data not shown). All muscle fibers in adult males express LM; the labeled probe is concentrated around cell nuclei (Fig. 3A), as has been described for other muscles (e.g., Huw *et al.*, 1977; Dix and Eisenberg, 1991). Female laryngeal muscle expresses LM only in some fibers that are often clustered (Figs. 3B and 3C). Previous studies demonstrated that in females, laryngeal muscle is heterogeneous with respect to fiber size: large- and medium-sized fibers are predominantly fast twitch while smaller fibers are usually slow twitch (Sassoon *et al.*, 1987). We thus examined the size distribution of labeled cells in sections prepared by combined *in situ* hybridization/immunohistochemistry of adult female laryngeal muscle. A fiber was considered labeled if the number of silver grains over it exceeded 2 $\times$ background. About 20% of fibers were labeled with the LM probe. Of the labeled fibers, 17% were large, 79% were medium, and 4% were small. This size relationship is also apparent in the digoxigenin-labeled sections (Figs. 3B and 3C).

To assess the expression of LM in other muscles, anti-sense f3-500 was hybridized *in situ* to several skeletal and cardiac muscles of an adult male frog (Fig. 4). The dilator laryngis muscle showed strong expression of LM (Fig. 4A); labeling of other muscles did not exceed levels seen in sense controls (compare Fig. 4C to Figs. 4E, 4G, 4I, and 4K). No expression was present in the geniohyoideus internus, an extrinsic laryngeal muscle (Figs. 4E and 4F), nor in the sternoradialis (Figs. 4I and 4J), an androgen-regulated muscle that participates in the male clasp reflex (Rubinstein *et al.*, 1983). In addition, we did not detect expression of LM in tibialis anterior (Figs. 4G and 4H), a predominantly fast muscle (Martin Jones, personal communication) nor in cardiac muscle (Figs. 4K and 4L), known to contain an androgen-regulated MHC in rats (Morano *et al.*, 1990). No signal above background was observed in three additional muscles: semimembranosus, iliofibularis, and flexor carpi radialis (data not shown). The *in situ* hybridization studies were repeated using clone H11 as a riboprobe. The pattern of expression of this larger, more 5' fragment, entirely confined to the coding region, was indistinguishable from that of f3-500. Only laryngeal muscle was labeled.

Expression of the Male-Specific MHC Isoform during Laryngeal Development

We examined LM expression during postmetamorphic development of male and female larynges. Male laryngeal muscle was studied at the beginning of fiber type conversion (PM2), at an intermediate stage (PM3), and when all fibers are fast twitch (PM6). Northern analysis detected strong expression of LM in males at PM2 (Fig. 5A). The level of expression increased at PM3 and was then maintained through adulthood (PM6). Expression of LM in female laryngeal muscle was considerably lower than in male muscle at all developmental stages examined (Fig. 5B). The female temporal pattern of expression differed dramatically from that observed in males. Maximal expression of LM was observed at PM2 and then declined to barely detectable levels in the adult (Fig. 5C; a longer exposure of the same blot shown in Fig. 5B).

To further characterize the developmental LM expression, antisense riboprobes were hybridized *in situ* to laryngeal sections of male and female frogs at different stages of development (Fig. 6). RNA probes corresponding to the sense strand were used as a control for nonspecific binding; no signal was ever observed with the sense strand (see Figs. 4C and 4D). After metamorphosis was complete (PM0), male laryngeal muscle showed levels of LM expression well above background (Fig. 6A) and expression was much higher than that in the female laryngeal muscle (Fig. 6B). At PM1, LM expression in male laryngeal muscle became more intense and uniform (Fig. 6C). Expression was further increased at PM2 (Fig. 6E), reached the highest level at PM3 (Fig. 6G), and was then maintained through PM5 (Fig. 6I). In female laryngeal muscle, expression of LM increased between PM0 and PM2 (Figs. 6B, 5D, and 5F), but then decreased (Figs. 6H and 6J). A patchy, nonuniform distribution of label was seen throughout postmetamorphic development in females. We think that the clustering of fibers expressing LM (see Fig. 3B) accounts for the patchy distribution of labeled fibers in autoradiograms of female muscle. From PM2 on, all fibers expressed LM in males.

Androgen Regulation of LM Expression

To study the effect of androgen on the expression of LM, PM2 female and male frogs were treated with DHT for 3 weeks and their larynges were processed for *in situ* hybridization. Androgen treatment increases LM expression in PM2 females (Figs. 7A and 7C). DHT-treated female laryngeal muscle expresses LM with a uniform pattern of hybridization (Fig. 7C) indistinguishable from that of the male (Fig. 7D). DHT treatment does not increase LM expression in PM2 males (Fig. 7B) but does induce muscle hypertrophy (Fig. 7D); hypertrophy is also apparent in DHT-treated PM2 female laryngeal muscle (Fig. 7C) and has been reported previously in response to androgen at this stage (Sassoon and Kelley, 1986; Sassoon *et al.*, 1987; Tobias *et al.*, 1991b).

DISCUSSION

The Laryngeal Clones Are Derived from an MHC mRNA

We have isolated MHC cDNA clones that hybridize preferentially to male laryngeal muscle RNA. Four lines of evidence indicate that these clones are derived from a laryngeal myosin heavy chain gene: (1) They were isolated using an MHC probe from thigh muscle of *X. laevis*. (2) They are expressed only in muscle fibers. (3) They hybridize to a 6.2-kb transcript, a size similar to that of other MHC mRNAs. (4) They share extensive homology at the nucleotide and amino acid levels to several vertebrates' MHCs.

LM Expression and Sexually Dimorphic Twitch Types in the *X. laevis* Larynx

A striking feature of laryngeal muscle in adult males is that it is composed entirely of fast twitch, fatigue-resistant muscle fibers (Sassoon *et al.*, 1987). We have speculated that this

unusual homogeneity is related to the vocal demands placed on males by the production of courtship song, sporadic but prolonged bouts of rapid (70 Hz) muscle contractions (Tobias and Kelley, 1987). Analogy to other specialized muscles (e.g., mammalian extraocular, Wieczorek *et al.*, 1985, and jaw muscles, Rowleson *et al.*, 1981) suggested that the *X. laevis* larynx might express a unique myosin. We have identified a candidate gene, LM. Expression of LM appears limited to intrinsic muscles of the larynx that participate in song production. No expression was detected in seven other skeletal and cardiac muscles including other fast twitch muscles and other androgen-regulated muscles.

Every muscle fiber of the male larynx is fast twitch (Sassoon *et al.*, 1987; Tobias *et al.*, 1991a) and, as we show here, every muscle fiber expresses LM. Thus, LM is a candidate fast twitch myosin in male larynx. In mammals, a number of myosin heavy chains have been identified in fast twitch muscles and individual fibers can express more than one MHC (reviewed in Pette and Staron, 1990). It is thus possible that LM is coexpressed with another myosin or myosins, which might also influence or even predominate in control of physiological twitch type.

Female laryngeal muscle contains a mix of fast and slow fiber types (Sassoon *et al.*, 1987; Tobias *et al.*, 1991a). We have shown that the presence of slow twitch fibers provides a powerful constraint on the rate of muscle contraction and prevents females from producing the rapid trills of male song (Tobias and Kelley, 1987). In females, the sizes of laryngeal muscle fibers are correlated with their twitch properties: slow twitch fibers are smaller and fast twitch fibers are larger (Sassoon *et al.*, 1987). The present study indicates that these smaller, and thus slow, fibers do not express LM. Only a minority of the larger, fast fibers of the female larynx express LM. We conclude that while LM-expressing fibers of the female are fast, not all fast fibers express LM. Other fast twitch MHCs must be expressed in the female larynx. The functional consequences of LM expression, which we have demonstrated here only at the mRNA level, await future studies.

Sexual Differentiation of Fiber Type and LM Expression during PM Development

The fully masculine larynx of adult males develops from an initially feminine phenotype (Tobias *et al.*, 1991a). At the end of metamorphosis (PM0), the larynx of both sexes contains a mix of fiber types; females maintain this mix while fibers in males gradually transform to all fast twitch beginning at PM2. The number of axons in the laryngeal nerve, however, becomes dimorphic during metamorphosis, and this number may be altered by androgen treatment (DHT) or by antiandrogen treatment (flutamide) at tadpole stage 54 (Robertson *et al.*, 1991), suggesting that the levels of androgens are different in male and females well before the end of metamorphosis. LM expression has a sex-specific developmental history: at PM0 expression is already higher in male than in female muscle and every muscle fiber in male larynx expresses LM. The increased level of LM expression in males at PM0 may be attributable to prior androgen secretion. The sexually dimorphic pattern of LM expression suggests that it could contribute to fiber-type conversion in males. LM expression seen at PM0, however, precedes masculinization of fiber type not seen until after PM2. The delay between LM expression and fiber conversion may reflect requirements for the accumulation of a critical mass of LM protein before fiber type conversion.

The adult female larynx contains, like its juvenile counterpart, a mix of fast and slow twitch fibers (Sassoon *et al.*, 1987). If LM expression also contributes to fiber type in juvenile female larynx, the two should be temporally correlated. Expression of LM in juvenile females peaks at PM2 and then declines. Between PM1 and PM6, the relative proportion of fast twitch fibers in the female larynx also decreases (Sassoon *et al.*, 1987). As only some fibers express LM, this change could be due either to diminished expression within individual fibers or to a decrease in the percentage of fibers expressing LM.

Androgen Regulation of MHC Expression in Developing Muscle

Masculinization of fiber type expression in the larynx requires testicular androgen secretion (Tobias *et al.*, 1991b). Laryngeal muscle of juvenile females can be converted to fast twitch by administration of testosterone (Sassoon *et al.*, 1987; Tobias *et al.*, 1991b) or dihydrotestosterone (Tobias *et al.*, 1992). We show here that dihydrotestosterone treatment of juvenile female frogs increases LM expression by, at least in part, inducing expression in all muscle fibers. The correlation between DHT-induced LM expression and DHT-induced fiber type conversion again suggests that LM may participate in fiber type conversion in the larynx. DHT treatment does not increase LM expression in PM2 males probably because all muscle fibers of males already express LM at PM2. It will be of interest to determine whether LM expression in PM2 males, already maximal, requires gonadal androgen for maintenance. The maintenance of all fast twitch muscle fibers in the adult larynx does not appear to require continued exposure to androgen (Tobias and Kelley, 1987; Tobias *et al.*, 1991b; Watson and Kelley, 1992), and it will thus also be of interest to examine LM expression in castrated adult males and androgen-converted adult females after testosterone treatment is discontinued.

Taken together, our evidence suggests that LM in *Xenopus laevis* may encode a laryngeal-specific, androgen-regulated MHC isoform responsible for the fast twitch features of male muscle.

Androgen-Regulated MHC Isoforms and Development of Muscle Fiber Types

Results from DHT-treated juvenile females, together with sex differences throughout development, indicate that LM may be an androgen-regulated MHC isoform in *X. laevis* larynx. Androgen-regulated contractile protein isoforms have been identified in another sexually dimorphic muscle, the temporalis of guinea pigs (Lyons *et al.*, 1986). Adult females express a fast-red isoform and adult males a fast-white isoform (Gutmann *et al.*, 1970). At birth, both sexes express the adult female isoform; at puberty, the male temporalis begins to transform and 120 days later contains predominantly fast-white MHC (Lyons *et al.*, 1986). Other muscles in guinea pig also express this fast-white isoform and expression in these muscles (masseter, psoas, and longissimus dorsi) is not sexually dimorphic. The guinea pig temporalis muscle requires the continued presence of androgen to express the fast-white isoform since males castrated at 120 days after puberty revert to the feminine isoform. Adult females can be induced to express the fast-white isoform with testosterone treatment.

The ontogenetic and hormonal control of LM in *X. laevis* differ in several interesting ways from the androgen-sensitive guinea pig temporalis described above. First, LM appears to be larynx specific and available evidence on the fast-white MHC suggests that it is not muscle specific (Lyons *et al.*, 1986). Second, the fast-white MHC requires continued exposure to androgen while our histochemical and physiological analyses in *X. laevis* suggest that, once converted, a fiber does not revert to slow twitch when androgen is withdrawn (Sassoon *et al.*, 1987; Tobias and Kelley, 1987; Tobias *et al.*, 1991b). The permanent change in MHC expression induced by androgen exposure and the muscle-specific expression of LM may be related. We have speculated that developing *X. laevis* laryngeal muscle contains specialized myoblasts whose maintenance and proliferation is due to androgen secretion in males (Kelley *et al.*, 1989). If these myoblasts have the potential to produce LM, they could permanently change MHC expression in existing fibers through fusion (as is known to occur in mouse-human heterokaryons; Blau *et al.*, 1986). This mechanism could impart a stability to LM expression that a more conventional scenario, transcriptional activation of LM by androgen (as occurs in a cardiac MHC; Morano *et al.*, 1990, and perhaps also in guinea pig temporalis), might not.

LM Can Be Used to Explore the Molecular Basis of Androgen-Directed Laryngeal Development

Laryngeal muscle expresses high levels of androgen receptor during postmetamorphic development (Kelley *et al.*, 1989; He *et al.*, 1990), suggesting androgen could be acting directly on laryngeal muscle. However, nerve activity also influences myosin expression (Jolesz and Sreter, 1981) and laryngeal motor neurons also express androgen receptor at these developmental stages (Gorlick and Kelley, 1986); thus, androgen could regulate LM expression via nerve activity. We have explored this question by examining androgen-induced fiber type conversion in unilaterally denervated laryngeal muscle (Tobias *et al.*, 1992). Androgen administration converts laryngeal muscle fibers of PM2 male and female frogs to entirely fast twitch regardless of the state of innervation. Thus, androgen does not act through the nerve but probably acts directly on laryngeal muscle to control MHC expression. The LM gene provides us with starting material for further molecular exploration of mechanisms underlying the sexual differentiation of this vocal neuromuscular system.

Acknowledgments

This research was supported by NIH Grants 19949 and 23684. We gratefully acknowledge the helpful comments of Gabriel Aisemberg, Michael Cohen, Melanie Marin, and James T. Watson, the excellent photomicroscopic assistance of John Robertson, and the generous gifts of *Xenopus* myosin clones from Gary Radice and a myosin antibody from Martin Jones.

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Fig. 1.

Northern blot analysis of total RNA isolated from pooled laryngeal muscle from female (F) or male (M) frogs. (A) Hybridization was performed for 18 hr at 42°C with a ^{32}P -labeled *PstI/EcoRI* fragment of F3 (f3-500). Exposure time was 2 hr. (B) Verification that each lane of the blot contains similar amounts of RNA by reprobing the membrane with a cardiac actin probe (Dworkin-Rastl *et al.*, 1986). Exposure was overnight.

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Lys Leu Glu Thr Arg Val Arg Glu Leu Glu Asn Glu Leu Asp Ser Glu Gln Lys
AAA CTA GAA ACC AGG GTC CGA GAA TTG GAA AAT GAG CTG GAC AGT GAA CAG AAA

Arg Gly Ile Glu Ala Ile Lys Gly Val Arg Lys Tyr Glu Arg Arg Val Lys Glu
CGC GGT ATT GAA GCC ATA AAA GGT GTT CGC AAA TAT GAG AGA AGG GTG AAG GAA

Leu Thr Tyr Gln Thr Glu Glu Asp Arg Lys Asn Val Leu Arg Leu Gln Asp Leu
CTC ACT TAC CAG ACT GAA GAA GAC AGA AAG AAT GTT CTG AGA CTT CAG GAC CTG

Val Asp Lys Leu Gln Leu Lys Val Lys Ala Tyr Lys Arg Gln Ala Glu Glu Ser
GTG GAC AAA CTT CAG CTG AAG GTT AAA GCC TAC AAA AGA CAG GCC GAG GAG TCT

Asp Glu Gln Ala Asn Ala His Leu Ser His Phe Arg Lys Val Gln His Glu Leu
GAT GAA CAA GCC AAT GCC CAC CTG AGC CAT TTT AGG AAA GTG CAG CAT GAG CTT

Glu Glu Ala Glu Glu Arg Glu Leu Ile Ser Leu Asn Leu Lys *
GAG GAA GCA GAA GAG AGA GAG CTG ATA TCG CTG AAT CTC AAG TAA ATA AGC TGA

GGG CAA AAA GCC GCG ATA TCG GAG GCA AGA AAG AAT CTG AAG AGT GAA GAC CTG

TCT GGA CAG AAG AAA GAG GAA TGG ACA CTT TTC ATA TTT AAT ATC CCT TAT CTG

AAT CCC CTT ACT TAT TTT CCT TTG

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Fig. 2.

Nucleotide and derived amino acid sequences of the f3-500 MHC cDNA clone. The sequence is aligned as in the chicken embryonic MHC cDNA clone 251 (Kavinsky *et al.*, 1983). * indicates the termination codon. Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. L01495.

Fig. 3. Expression of LM in adult larynx of *X. laevis* as determined by *in situ* hybridization. Digoxigenin-labeled, antisense strand of f3-500 was used as a riboprobe. (A) Male laryngeal muscle. (B and C) Female laryngeal muscle. Big arrows indicate nuclei surrounded by the digoxigenin-labeled myosin probe. Small arrows indicate unlabeled nuclei. Scale bar: A and B, 30 μm ; C, 12 μm .

Fig. 4. LM expression in different skeletal and cardiac muscles of the frog *Xenopus laevis*. Dark-field (left) and the corresponding bright-field (right) views are shown. All sections were hybridized using ^{35}S -labeled antisense strand except for C and D where the sense strand of f3-500 was used. A–D, dilator laryngis; E and F, geniohyoideus internus; G and H, tibialis anterior; I and J, sternoradialis; K and L, cardiac muscle. Scale bar: 100 μm .

Fig. 5.

Northern analysis of laryngeal muscle RNA from developing frogs. Lanes 2, 3, and 6 are from postmetamorphic stages 2, 3, and 6, respectively. Hybridization conditions are as described in the legend to Fig. 1. The blots were stained with ethidium bromide to verify that each lane contained similar amounts of RNA ($5 \mu\text{g}$). Blots A and B were exposed overnight; blot C is a 72-hr reexposure of the blot shown in B.

Fig. 6.

LM expression during postmetamorphic laryngeal development as determined by *in situ* hybridization. Antisense probe is as described in the legend to Fig. 4. No signal was ever observed with the sense strand (see also Figs. 4C and 4D). Photomicrographs were prepared using dark-field illumination. Left panels show hemisections of male larynx; right panels show hemisections of female larynx. A and B, postmetamorphic stage (PM) 0; C and D, PM1; E and F, PM2; G and H, PM3; I and J, PM5. Note that hybridization signal is confined to muscular tissue and is not seen in cartilage or connective tissue (e.g., left-hand portion of A and B). Scale bar: 100 μ m.

Fig. 7. LM expression in PM2 female and male frogs treated with DHT for 3 weeks as determined by *in situ* hybridization. Antisense probe is as described in the legend to Fig. 4. A and B, control PM2 females and males, respectively, raised without hormone treatment for 3 weeks. C and D, DHT-treated females and males, respectively. Scale bar: 100 μ m.