Published in final edited form as:
Laryngoscope. 2008 March ; 118(3): . doi:10.1097/MLG.0b013e31815c1a93.

Quantification of Myosin Heavy Chain RNA in Human Laryngeal Muscles: Differential Expression in the Vertical and Horizontal Posterior Cricoarytenoid and Thyroarytenoid

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Abstract

Background—Human laryngeal muscles are composed of fibers that express type I, IIA, and IIX myosin heavy chains (MyHC), but the presence and quantity of atypical myosins such as perinatal, extraocular, IIB, and α (cardiac) remain in question. These characteristics have been determined by biochemical or immunohistologic tissue sampling but with no complementary evidence of gene expression at the molecular level. The distribution of myosin, the main motor protein, in relation to structure-function relationships in this specialized muscle group will be important for understanding laryngeal function in both health and disease.

Objectives—We determined the quantity of MyHC genes expressed in human posterior cricoarytenoid (PCA) and thyroarytenoid (TA) muscle using real-time quantitative reverse-transcriptase polymerase chain reaction in a large number of samples taken from laryngectomy subjects. The PCA muscle was divided into vertical (V) and horizontal (H) portions for analysis.

Results and Conclusions—No extraocular or IIB myosin gene message is present in PCA or TA, but IIB is expressed in human extraocular muscle. Low but detectable amounts of perinatal and α gene message are present in both of the intrinsic laryngeal muscles. In H-and V-PCA, MyHC gene amounts were β greater than IIA greater than IIX, but amounts of fast myosin RNA were greater in V-PCA. In TA, the order was β greater than IIX greater than IIA. The profiles of RNA determined here indicate that, in humans, neither PCA nor TA intrinsic laryngeal muscles express unique very fast-contracting MyHCs but instead may rely on differential synthesis and use of β, IIA, and IIX isoforms to perform their specialized contractile functions.

Keywords

Human laryngeal muscle; myosin heavy chain RNA; quantitative reverse-transcription polymerase chain reaction; extraocular myosin; myosin IIB

INTRODUCTION

The intrinsic laryngeal muscles coordinate vocal fold movements during respiration, deglutition, and phonation. Of this muscle group, the thyroarytenoid (TA) is one of several adductors, whereas the posterior cricoarytenoid muscle (PCA) is the lone abductor of the vocal folds. In humans, the TA muscle is composed of two functional compartments, a

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medial portion whose fibers intertwine with connective tissue elements of the vocal ligament folds and a lateral portion whose structure more resembles a muscle belly. The medial portion of the TA is sometimes referred to as the "vocalis portion" because it alters vocal fold tension and relaxation during speaking or singing. The lateral portion of the TA is termed the "muscularis portion" because it produces gross movements in the vocal folds.[1] The human PCA muscle is composed of distinct horizontal (H) and vertical (V) fan-shaped portions[2,3] that, respectively, coordinate rotation and sliding of the arytenoid cartilage for opening the glottis during respiration and vocalization.

Finer descriptions of the functional specialization of intrinsic laryngeal muscles have been largely teleologic, based primarily on animal experiments, the results of which, given the differences in anatomy, innervation, and composition of myosin isoforms, may not directly apply to the condition in humans. One of the best approaches for understanding the function of these muscles has been tissue level investigations of fiber type composition and contractile protein expression found in the different fiber types. Both the TA and PCA contain typical slow (type I) and fast (type IIA and IIX) skeletal muscle fiber types, which may be identified by myofibrillar ATPase histochemical analysis and iinmunohistochemistry using myosin heavy chain (MyHC)-specific antibodies.[4,5] The TA is more abundant in type II fibers given the need for rapid contractions that position the vocal folds for initiation and variation of speech and singing. Given the functional diversity of human PCA muscle, the fiber type composition differs significantly among its two principal portions. The H belly, which is more active during inspiration, contains predominately type I fibers, whereas the V belly, which is more active during voice production, has a relatively equal distribution of type I and type II fibers.[4] The PCA, TA, and other intrinsic laryngeal muscles belong to a larger subset of skeletal muscle generally termed specialized cranial muscles.[5] Cranial muscles develop from populations of mesodermal cells, which are separate from the somatic lateral plate mesoderm that forms muscle of the axial skeleton.[6] Physiologic adaptation for performance of highly specialized motor tasks is associated with these developmental differences, as exemplified by the muscle-specific expression of MyHC genes that include the extraocular myosin (MyHC-EO) in muscles associated with eye movement[7,8] and the type II masticatory myosin (MyHC-IIM) in jaw-closing muscles.[9] Similar to other cranial muscles, laryngeal muscles may express atypical myosins such as perinatal and α-cardiac and contain fibers that co-express myosins. Given the need for rapid contraction, the possibility also exists that MyHC-IIB (the fastest contracting myosin typically found in type II fibers of the limb muscles of smaller mammals) might be expressed in the intrinsic laryngeal muscles.

Evidence of the expression of another MyHC specific to laryngeal muscle fibers was first suggested by biochemical isolation of an additional MyHC isoform.[10] In the absence of an MyHC–EO-specific antibody at the time to verify its identity, the protein species in rat laryngeal muscle was preliminarily considered to be an atypical myosin expression and designated type "II-L" or type II laryngeal. Subsequently, molecular techniques have been used to demonstrate expression of the MyHC-EO gene in rabbit laryngeal muscle,[11] which strongly suggests that the "II-L" myosin in rat laryngeal muscle is also the MyHC-EO isoform species. However, no MyHC-EO has yet been detected in human intrinsic laryngeal muscles.[12] Furthermore, there currently exists no evidence for novel MyHC isoforms that are specific to this muscle group.

Because most studies have used protein-based methods to characterize myosin composition and fiber types in human laryngeal muscles, elucidation of corresponding RNA species is important to more fully demonstrate the extent of MyHC expression at the gene level in these muscles. We have conducted a genetic analysis of muscle myosins using probes for major myosin RNA species that include β, which encodes the (β-cardiac isoform that is

predominate in slow type I fibers, and IIA and IIX, which determine the fast isoforms that comprise type II fibers. Additional probes for less abundantly expressed myosins were also used, including α, which specifies the α-cardiac isoform primarily found in "the atrium, perinatal," which encodes the developmental perinatal (or neonatal) isoform, extraocular, which is usually specific to eye muscle, and IIB, a fast-contracting myosin that is only selectively expressed in humans.

MATERIALS AND METHODS

Subjects and Tissues

Laryngeal muscles were obtained with approval of the university internal review board (IRB# 991280) from 15 patients 45 to 81 years of age (average age, 65 yr) undergoing total laryngectomy. Only subjects with normal vocal fold mobility, assessed prior to surgery, were included. At surgery, right and left PCA and TA muscles that were free of gross pathology were dissected from excised larynges. Availability of full sets of the laryngeal muscles, which included TA and right and left sides of both bellies of the PCA, was limited because of harvesting for pathology. During collection, obtainable PCA muscles were routinely divided into V and H bellies, and TA muscles were sampled from any available area that did not interfere with pathologic evaluation. For this reason, the number of TA samples from the subject group was limited in comparison with PCA samples. Infrahyoid muscle (IFH) was also sampled during laryngectomy and used as a "standard muscle" control. Biopsies of limb vastus medialis and extraocular internal oblique already available from previous work were also used for comparative purposes.

RNA Isolation and Reverse-Transcription Polymerase Chain Reaction

The average weights for the frozen muscle biopsies were 190 ± 27.2 mg for H-PCA, 205 ± 28.7 mg for V-PCA, and 128 ± 31.0 mg for TA. RNA was isolated from the muscle biopsies with TRIzol reagent (Invitrogen, Carlsbad, CA) followed by digestion with 10 U DNase I and heating at 65°C to inactivate enzyme. RNAqueous reagent (Ambion, Inc., Foster City, CA) was added to the sample digests, and RNA was purified according to the manufacturer's specifications and quantified by absorbance at A_{260}. The average recoveries of total muscle RNA were 278 ng/mg for H-PCA, 272 ng/mg for V-PCA, and 224 ng/mg for TA. Human atrium RNA was obtained from Ambion, Inc. for authentication of α-myosin expression. MyHC RNA was quantified by reverse-transcription polymerase chain reaction (RT-PCR) using Titanium One-Step RT-PCR kits (Clontech Laboratories, Mountain View, CA) that were supplemented with 0.4 μmol/L oligo dT(18 mer), 20 pg/μL random hexamer primers, and SYBR Green (Molecular Probes, Inc., Carlsbad, CA). Oligonucleotide PCR primers for MyHC-β, α, IIA, IIX, IIB, perinatal, and MyHC-EO gene message (Table I) were selected from GenBank mRNA sequences using Oligo 6 software and synthesized by a commercial source (Invitrogen). Qualitative analyses were first performed to determine the presence and general amounts of myosin isoform RNA, including MyHC-EO and IIB, in the laryngeal muscles. Thereafter, myosin RNA from samples was quantified in duplicate, 30 ng aliquots of total muscle RNA using a standard protocol for reverse transcription and PCR in all assays (Table II). Input muscle RNA and amplified complementary DNA were quantified from standard curves in each assay using QuantumRNA (Ambion, Inc.) 18S RNA Universal Standards (315 bp) and a plasmid (323 bp) DNA internal standard. RNA and DNA values were calculated at maximum amplification efficiency, with a slope within the range of −3.30 to −3.32 plotted for each standard curve. With use of analyses of variance (ANOVA), no statistical significance was detected for values of 18S RNA between samples taken from the different muscle types used in RT-PCR assays ($P > .734$). Amplification products were routinely verified by electrophoresis in 3% NuSieve 3:1 agarose gels containing 0.5 μg/mL ethidium bromide. Differences in quantities of myosin message in extracts of the several

muscle types were tested for significant mean differences using ANOVA for a between or between-within case design.

RESULTS

MyHC Gene Expression in Human PCA and TA

RT-PCR analyses were first performed to qualitatively determine whether RNA for the MyHC-EO isoform is present in human intrinsic laryngeal muscles. Agarose gel electrophoresis of RT-PCR products showed that MyHC-EO was expressed in a control sample of human extraocular muscle but not in vastus medialis or laryngeal PCA and TA muscles (Fig. 1). A similar analysis for the presence of MyHC-IIB message showed amplification of product in human extraocular muscle but no amplification in either compartment of human PCA, TA, and sartorius muscles (Fig. 2).

Real-time RT-PCR analyses were performed to quantify the differential expression of message for β, IIA, IIX, α, and perinatal myosins in the laryngeal muscles and in the IFH neck muscle. Quantifiable amounts of RNA for all the myosins, including perinatal and α-myosin, were found in most all the muscle samples. Because myosin RNA content between the right and left sides of the H and V bellies of the PCA were not different in subjects, data from those lateral pairs of muscles were pooled for analyses of their MyHC distribution. Perinatal MyHC RNA was present in very low amounts, and its muscle-specific distribution was compared separately from the other myosins.

ANOVA on the data shown in Figure 3 indicated that levels of RNA for the classes of myosins differed significantly across the four muscle types ($P = .002$), with the highest total expression seen in the PCA muscles and the lowest in the IFH neck muscle. The pattern of RNA quantities was β greater than IIA greater than IIX greater than α in all of the muscles except in the IFH, which expressed equally low amounts of MyHC-IIX and α. Values for the three major isoforms β, IIA, and IIX in the TA muscle were close to one another, resulting in proportionately more fast MyHC expression. Overall, expression of β-MyHC was highest in the two bellies of PCA muscle and significantly greater than in TA ($P < .010$) and approached significance in IFH ($P < .119$). MyHC-IIA was most abundant in the V-PCA at levels that were close to significantly greater than IFH ($P = .061$) and were significant over H-PCA and TA ($P < .004$ and .019, respectively). Expression of MyHC-IIX was slightly greater in the TA than in either compartment of the PCA muscles and significantly greater than in the IFH ($P < .034$). α-MyHC RNA was detected at low levels in most samples but undetectable from a few specimens, with no apparent correlation between its presence and muscle type. Levels of α were higher, however, in the all of the laryngeal muscles in comparison with the IFH.

Although detected in very low amounts, the presence of perinatal MyHC RNA in all but a few of the muscle biopsies was considered of interest because of the atypical expression of this developmental myosin isoform in adult tissues. Specific quantities of perinatal myosin were all less than 0.1 pg amplified DNA/ng 18S RNA (Fig. 4) in the separate muscle types, with more amounts detected in the PCA and TA than in the IFH, but the differences between laryngeal muscles and the neck were not statistically significant ($P = .371$).

When calculated as percentages of total MyHC RNA (Table III), β-MyHC comprised 71.6% of H-PCA, 55.4% of V-PCA, and 46.2% of TA. Fast MyHC gene expression, represented by the sum of RNA for IIA and IIX, accounted for 28.3% of H-PCA, 44.7% of V-PCA, and 53.8% of TA. In comparison, the IFH comprised 72.5% β and 27.5% IIA plus IIX myosin expression, of which only 3.8% was IIX.

DISCUSSION

The phenotypes and physical properties of laryngeal muscle fibers have been subjects of continuing interest and investigation.[13] Many studies have described myosin protein isoform expression in human laryngeal muscles using immunohistochemical and biochemical techniques but often with limited sample size.[14–16] This report is the first description of myosin gene expression in human laryngeal muscle from a relatively large group of subjects collected by the same laryngologist (C.R.). Understanding normal gene expression and modifications in expression is an important aspect of laryngeal skeletal muscle biology. We isolated muscle RNA to serve as a template for quantification of gene expression using an 18S RNA template as an internal standard between sets of muscle samples and different muscle types. A second set of standards using plasmid DNA was used to quantify amounts of amplified cDNA for the genes of interest. This served as a good approach given the need to determine expression values for five different genes in a large number of samples. The primers selected were specific for 3′ UTR regions of human MyHC genes that overlapped introns to verify that genomic DNA was not amplified during processing. Using these methods, we were able to determine at the molecular level that the extraocular muscle MyHC gene is not expressed in either PCA or TA muscle. Likewise, MyHC-IIB transcription is not found in the intrinsic laryngeal muscles. Notably, however, the IIB gene is expressed in human extraocular muscle similarly to that seen in specimens from rabbit,[17,18] cow,[19] and dog.[20] In the latter two reports, both MyHC-IIB RNA and protein were detected in the vocalis portion of canine TA muscle, whereas no expression of IIB was found in bovine laryngeal muscles. We have previously reported that MyHC-IIB RNA is detectible by in situ hybridization in human masseter muscle, but its translated protein is not seen,[21] so that expression of IIB myosin is complex and apparently regulated in a species- and muscle-specific manner that also may use posttranscriptional modification. Quantifiable small amounts of *α* and perinatal RNA were present in most all laryngeal muscle samples. Expression of perinatal myosin in the laryngeal muscles significantly exceeded that of control strap muscle of the neck. This overall comparative approach will be useful in future studies to determine variations in gene expression that occur in development or during disease or clinical interventions.

Typically, values of MyHC content in laryngeal muscle have been reported as a percent distribution of the total based on arbitrary units from densitometry of bands seen by gel electrophoresis[22] or from Western blots.[23] The real-time quantification of RNA by RT-PCR used here enables not only the comparison of the relative distribution in muscles but also the specific values for each MyHC expressed. We found the hierarchy of MyHC gene expression to be *β* greater than IIA greater than IIX in both H-PCA and V-PCA muscles, but the distribution of fast largest and strongest units, containing IIB fibres were recruited last (Burke *et al.*, 1971). All fibre types belonging to the same motor neuron were found to be histochemically similar (i.e. units were composed of all type I, IIA, or IIB fibres and never a mixture of different fibre types; Burke *et al.*, 1973). The same was true for human limb muscles in an interesting study conducted by (Garnett *et al.*, 1979). Even in rapid voluntary contractions in man, fast motor units cannot be preferentially recruited (Desmedt and Godaux, 1977).

Another important finding from the glycogen depletion technique was an understanding of the physical location of motor units in the muscle organ. Motor unit territory is variable in species and specific muscles since these differences are performance related. It is also the case in healthy muscle that fibres of many different motor units occupy the same area of muscle and are intermixed, giving a typical moasic pattern of differing fibre types with histochemical staining (Figure 5). Fiber type clumping, adjacent fibres of the same type and from the same motor unit is indicative of pathological conditions, such as denervation or re-

innervation (Karpati & Engel, 1968). Such conditions occur, for example, from sprouting of a single motor neuron, which establishes endplates on regenerating fibres in the same area.

Motor unit characteristics are described in several ways that include: the size of the fibres in the unit; the number of fibres in the unit; the motor unit territory (area and position the unit occupies relative to the total muscle); the force produced by motor unit contraction; and finally the speed of shortening of the fibres in the motor unit. Muscles that require fine, precise motor control usually have many motor units with a small number of fibres in the unit. The best example is the human extra-ocular muscles used in positioning the eye. They are estimated to have 2970 motor units with 9 fibres in each unit (Feinstein *et al.*, 1955). The extra-ocular muscles also have a large representation in the motor cortex (Cushid, 1976). As an example of a relatively large human muscle which requires less precise movement the biceps brachii muscle is estimated to have 3552 motor units with 163 fibres in each unit (Christensen, 1959). The human temporalis is estimated to have 1331 motor units and 936 fibres in each unit and the masseter to have 1452 motor units and 640 fibres in each unit (Carlsöö, 1958). The masseter muscle does have a large representation in the motor cortex (Cushid, 1976) and is known to exhibit very fine movement in some of its functioning, so the size of its motor units were surprising. Since glycogen depletion experiments are not possible in living humans, investigators began applying bite force and electrophysiological measurements to human jaw-closing muscles to further describe motor units. Stalberg and Eriksson (1987) described some of the units in human masseter using these techniques and found most motor unit territory to be relatively small (from 0·6 to 4·5 mm. There were only a few motor units with very large territory in the range of 9·1–12·5 mm. The small motor units were confined to limited areas of masseter, but the few large motor units tended to span almost the whole muscle cross section. Large motor units spanning nearly the whole area of a limb muscle, to the best of our knowledge, have never been found in other studies (Buchthal and Schmall, 1980; Stalberg *et al.*, 1976). It is possible that the small motor units in masseter are used for fine motor functions and the very large motor units to stabilize the entire masseter muscle for balance or when maximal force is necessary. The electrophysiological study by Stalberg and Eriksson (1987), however, is in sharp contrast to the general description given by Carlsöö, (1958), who produced his results from an anatomic study of the innervation ratio. Carlsöö attempted to estimate the total number of α-motor nerves and the total number of skeletal fibres in the entire temporalis and masseter muscles. Stalberg and Eriksson's (1987) description of masseter motor units contained only those recruited to produce low bite forces and do not represent all of the units in the muscle. When similar EMG techniques were combined with magnetic resonance imaging of 162 motor units in human masseter, most of these units were found positioned between tendons and only a few (10 per cent) crossed tendons (Tonndorf and Hannam, 1994). This confirmed the idea that almost all masseter motor units are located in discrete compartments that may produce movement in a variety of directions, and some units cross tendons to stabilize compartments when necessary.

Animal experiments on motor unit territory and recruitment pattern do not necessarily provide answers for the way in which units work in human jaw-closing muscles given the very odd human phenotypes for masticatory skeletal muscle fibres. The only animal model which comes close to representing human jaw-closing muscle fibres is the rabbit, since its fibres express α-cardiac myosin heavy chain (Sciote and Kentish, 1996) and there is heterogeneous expression of myosin heavy chains in some individual fibres (Bredman *et al.*, 1991). In glycogen depletion experiments, which show the three-dimensional relationship of the total motor unit area to the total masseter muscle area for rabbit (Weijs *et al.*, 1993), the 11 motor units studied were restricted to small portions of masseter with total fibre number ranging from 40 to 424. The surprising finding in nearly half the motor unites investigated, however, was that not all fibres were of the same type. This observation directly contrasts

Burke *et al.*'s (1973) finding in limb muscle that all motor units are homogeneous for fibre type. Those motor units found to be heterogeneous for fibre type contained a combination of fibre types, either α + IIA fibres or IIAB + IIA fibres. This data strongly suggests that in jaw-closing muscles of some mammals, fibres may be heterogeneous for myosin heavy chain isoforms and motor units may be heterogeneous for fibre type. However, the limited number of motor units examined in this study may not represent the whole muscle. Nevertheless, in general the α motor units had the smallest number of fibres and smallest territory, the IIA motor units had relatively much larger fibre number and territory, and the IIB units the largest fibre number and territory. Recruitment order of these unusual motor units was not described, but given anatomical description of unit size and myosin composition it is likely that rabbit masseter units are recruited in an orderly fashion from small to large and from slowest contracting to fastest contracting.

In human masseter, however, the recruitment of motor units has been investigated with electrophysiological techniques. The recruitment pattern is orderly for the size of the unit, but may not be orderly for the speed of shortening of the units. Yemm (1976, 1977) did find orderly recruitment for motor unit size and bite force, but found either no correlation between size and contraction speed, or a reverse correlation between size and contraction speed of some units. Yemm suggested that for contraction speed it is quite possible that faster motor units are recruited *before* slow motor units. Goldberg and Derfler (1977) produced results similar to that of Yemm in that there was an orderly recruitment of masseter motor units for size, but no correlation between size and speed of contraction. Both studies confirm the well documented results of fibre typing studies done on muscle sections which clearly show that the fast contracting fibres in human masseter are much smaller than the slow contraction type I fibres. It is also not surprising that a reverse correlation in motor unit recruitment for speed of shortening cannot always be found since myosin expression in masseter fibres is often very heterogeneous. Recent studies have used different EMG techniques to investigate human masseter motor unit recruitment (Scutter and Turker, 1998), but very little additional information regarding recruitment order and speed of contraction have been obtained.

The size principle was first proposed for gradually increasing isometric contraction, but muscle contraction in living animals is not always isometric. Even in muscles that can be studied for isometric contraction, the same motor unit may have different recruitment thresholds for flexion versus extension (ter Harr Romeny *et al.,* 1982). Muscles that move bone in various directions do have consistently different motor unit recruitment based on the direction of force production. (Thomas *et al.,* 1978; Desmit, 1977; Schmidt and Thomas, 1981) Such observations have often been termed 'task specific behavior of motor units'. Ericksson *et al.,* (1984) have described such behaviour in human masseter and English (1985) confirmed that not all the motor units in a muscle are active during contraction. The idea that there are subpopulations of motor neurons that will respond differentially to directional movement is an exception to the size principal for both jaw-closing and limb muscles, but for reasons different than fibre type composition of the muscle. Task specific behaviour is observed in multifasiculated muscles that do perform a variety of patterned movements (Freund, 1983). Such muscles also have very sophisticated afferent input to their motor neuron pools that help modify the excitability of motor neurons controlling the motor units (Kanda *et al.,* 1977; Luescher *et al.,* 1979). In the case of human masseter this afferent information is produced principally by highly complex muscle spindle arrangements (Rowlerson, 1990) and peridontal afferents from the tooth ligaments (Linden, 1990).

Acknowledgments

This work was supported by an RO1 grant to Dr. Sciote, NIDCD 5058-3.

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Fig. 1.

Muscle-specific expression of extraocular myosin heavy chains (MyHC). Human vastus medialis (b, f), extraocular (c, g), posterior cricoarytenoid (d, h), and thyroarytenoid (e, i). Classic 18S rRNA (489 bp; b to e). Extraocular MyHC RNA (243 bp; f to i). DNA standards: 25 bp (a) and 100 bp (j).

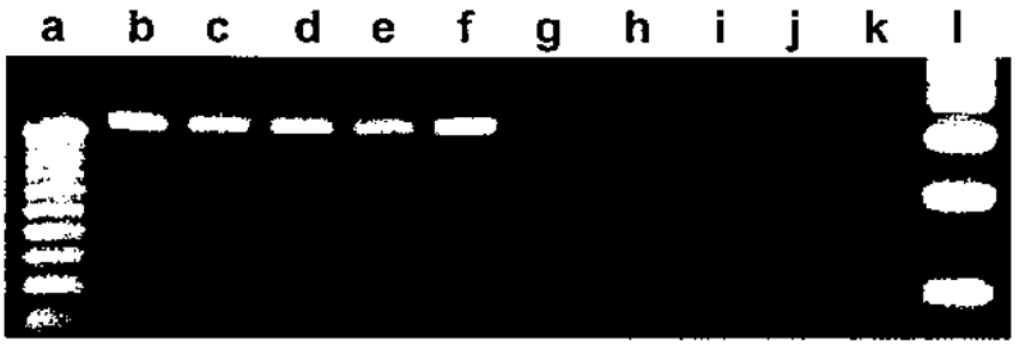

Fig. 2.

Muscle-specific expression of IIB myosin heavy chains (MyHC). Human extraocular (b, g), horizontal posterior cricoarytenoid (PCA) (c, h), vertical PCA (d, i), thyroarytenoid (e, j), and sartorius (f, k). Universal 18S rRNA (315 bp; b to f). IIB MyHC RNA (114 bp; g to k). DNA standards: 25 bp (a) and 100 bp (I).

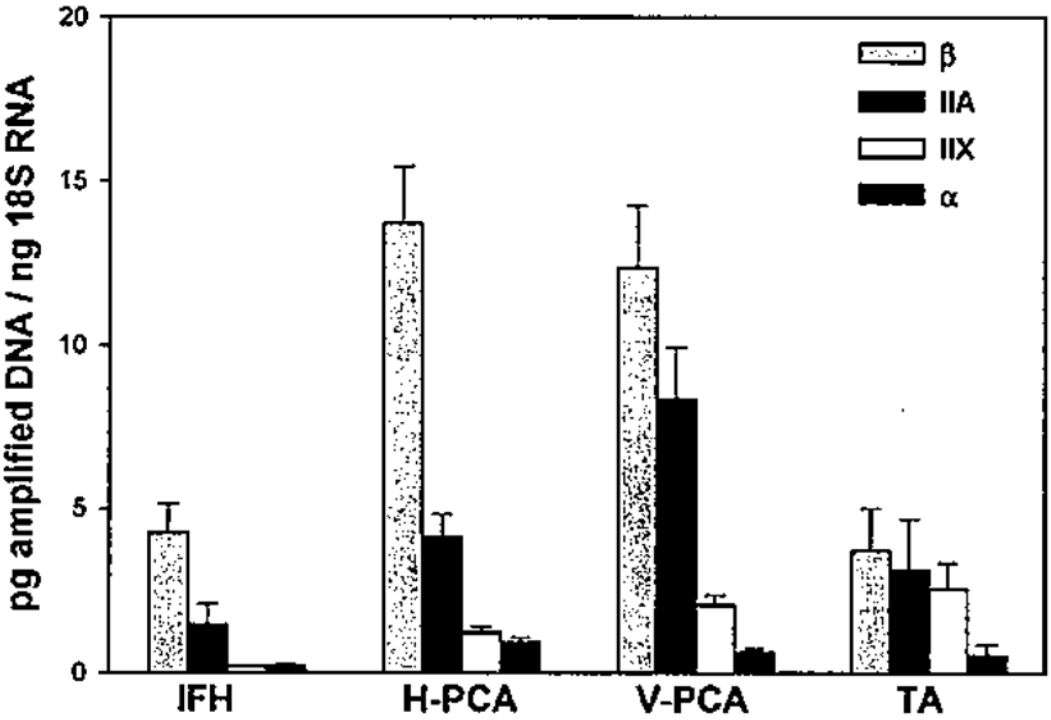

Fig. 3.

Myosin heavy chain (MyHC) RNA in infrahyold (IFH) and laryngeal muscles. Data are averages ± SE of MyHC-*β*, IIA, IIX, and *α* for IFH (n = 2), horizontal posterior cricoarytenoid (H-PCA) (n = 19), vertical (V)-PCA (n = 20), and thyroarytenoid (TA) (n = 6).

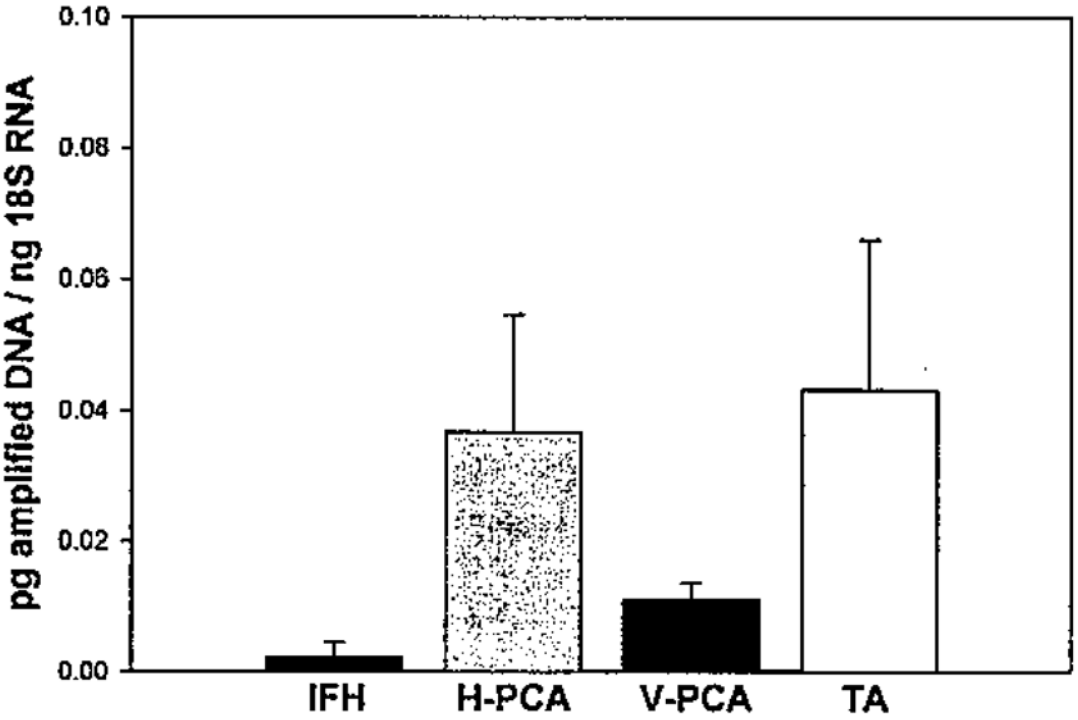

Fig. 4.

Perinatal MyHC RNA in infrahyoid (IFH) and laryngeal muscles. Data are averages of picograms of amplified DNA/ng 18S RNA ± SE for IFH (n = 2), horizontal posterior cricoarytenoid (H-PCA) (n = 19), vertical (V)-PCA (n = 20), and thyroarytenoid (TA) (n = 6).

TABLE I

Primers for Reverse-Transcription Polymerase Chain Reaction of Myosin Heavy Chain (MyHC) Genes.

MyHC	Sense Primer	Antisense Primer	Product Length (bp)
α	5′-AAGCTGCAACTGAAGGTCAAGG-3′	5′-CAGTGTCACTCCTCATCGTGCATT-3′	212
β	5′-AGACGGAGGAGGACAGGAAA-3′	5′-AGATCAAGATGTGGCAAAGCTACT-3′	270
IIA	5′-AAGGTCTCCATTTACAAGCTCACG-3′	5′-TTGGACACCTGTTCTACAGTCTGG-3′	238
IIX	5′-CTGCAAGCAAAGGTGAAATCCTA-3′	5′-CACATTTTGTGCATTTCTTTGGTC-3′	243
IIB	5′-CGGGAGGTTCACACAAAAGTCATA-3′	5′-CCTTGATATACA GGACAGTGACAA-3′	114
Perinatal	5′-GAAACATGACCGACGAGTAAAAG-3′	5′-CAGGTGTGTTTACTCTGCACTGAT-3′	292
Extraocular	5′-GAGTAACATAGAAAGAACGTGCCG-3′	5′-GTTTCTTCTTCCATTTGCCTCTTA-3′	243

TABLE II

Protocol for Reverse-Transcription Polymerase Chain Reaction of Myosin Heavy Chains.

Step	Function	Temperature (°C)	Time
1	Reverse transcription	50	60 min
2	1° denature	95	5 min
3	Cycle denature	94	10 sec
4	Anneal	62*	30 sec
5	Extend	72	1 min
6	Read plate 1	78	1 sec
7	Read plate 2	80	1 sec
8	Go to line 3, 35×	—	—
9	Final extend	72	10 min

* 65°C was used for reverse-transcription polymerase chain reaction of myosin heavy chain-IIB.