

Growth and Muscle Defects in Mice Lacking Adult Myosin Heavy Chain Genes

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Abstract. The three adult fast myosin heavy chains (MyHCs) constitute the vast majority of the myosin in adult skeletal musculature, and are >92% identical. We describe mice carrying null mutations in each of two predominant adult fast MyHC genes, IId and IId/x. Both null strains exhibit growth and muscle defects, but the defects are different between the two strains and do not correlate with the abundance or distribution of each gene product. For example, despite the fact that MyHC-IId accounts for >70% of the myosin in skeletal muscle and shows the broadest distribution of expression, the phenotypes of IId null mutants are generally

milder than in the MyHC-IId/x null strain. In addition, in a muscle which expresses both IId and IId/x MyHC in wild-type mice, the histological defects are completely different for null expression of the two genes. Most striking is that while both null strains exhibit physiological defects in isolated muscles, the defects are distinct. Muscle from IId null mice has significantly reduced ability to generate force while IId/x null mouse muscle generates normal amounts of force, but has altered kinetic properties. Many of the phenotypes demonstrated by these mice are typical in human muscle disease and should provide insight into their etiology.

MYOSIN heavy chains (MyHCs)¹ comprise a family of molecular motor proteins, eight of which are expressed in mammalian cardiac and skeletal muscle. Each is characterized by its time and site-specific expression pattern. There are two principal developmental isoforms expressed in embryonic and neonatal skeletal muscle (Bouvagnet et al., 1987; Periasamy et al., 1984), and four isoforms expressed in adult fast skeletal muscle (Weydert et al., 1983; Parker-Thornburg et al., 1992). One of these genes is exclusive to adult extraocular muscle (Wieczorek et al., 1985). Two isoforms are expressed in cardiac muscle (Gulick et al., 1991), one of which is also the slow skeletal muscle isoform (Rindt et al., 1993).

Members of the sarcomeric MyHC multi-gene family contain 39–40 exons (Mahdavi et al., 1986) and exhibit a striking degree of sequence conservation including the location of intron/exon boundaries among the isoforms as well as between such widely divergent species as rat and *Caenor-*

habditus elegans. The MyHC genes expressed primarily in skeletal muscle are located in a cluster on mouse and human chromosomes 11 and 17, respectively (Leinwand et al., 1983), while the two cardiac MyHC genes (α and β) are located on mouse and human chromosomes 14 (Saez et al., 1987; Weydert et al., 1985).

Myosin is the major component of thick filaments within the sarcomere. A single holomyosin molecule (~600 kD) is composed of two identical heavy chains (230 kD each), two phosphorylatable (regulatory) light chains and two alkali (essential) light chains. The COOH-terminal rods of the two heavy chains assemble into a coiled-coil, while their NH₂-terminal sequences fold to form two globular heads which constitute the motor domain.

Muscle contraction involves the coordinate action of nerve stimulus, ion transport, metabolic energy substrates and the proper functioning of the sarcomeric “engine” for its normal activity. Abnormalities in any of these individual constituents could conceivably result in the improper function of the assembled sarcomere. In this context, the myosin molecule is one of the more important structural components of the muscle sarcomere (representing ~40% of the myofibrillar protein) (Warrick and Spudich, 1987). While mutations in the major cardiac MyHC gene (MyHC- β) have been implicated in a dominant genetic heart disease,

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1. *Abbreviations used in this paper:* CSA, cross-sectional area; EDL, extensor digitorum longus; NADH-TR, NADH-tetrazolium reductase; MyHC, myosin heavy chain.

familial hypertrophic cardiomyopathy (Marian and Roberts, 1994), no diseases specific to skeletal muscle have yet been ascribed to MyHC mutations.

Most vertebrate skeletal muscles express multiple MyHC genes. The most striking examples of this are adult masseter muscle and extraocular muscle. The masseter expresses seven of the eight known muscle MyHC genes including both developmental isoforms, all three adult fast isoforms, the β -slow, and the α -cardiac. Extraocular muscle expresses, in addition to the seven genes described above, the extraocular isoform, MyHC-eo (Wieczorek et al., 1985; Pedrosa-Domellof et al., 1992).

The number of members in the myosin gene family and its complex temporal and spatial patterns of expression raise the question of whether each gene is functionally distinct or whether there is functional redundancy within the family. There are several lines of evidence that suggest functional diversity within the family. First, the orthologous MyHCs from different species are more related to each other than family members within a species (Weiss and Leinwand, 1996). Second, the contractile velocity of a muscle has been shown to largely correlate with its myosin content (Barany, 1967). While the enzymatic properties of the two cardiac MyHCs (α and β) have been shown to be quantitatively distinct, the biochemical properties of each skeletal myosin isoform are not yet known. Arguing against distinct functions for each gene are the common origin of vertebrate skeletal MyHC isoforms and their very high degree of conservation in genomic structure and amino acid sequence. This argument may be most relevant to the three adult fast isoforms which include the MyHC-IIa, -IIB, and -IId/x genes. These three genes are highly homologous and are broadly expressed in skeletal musculature. The fast IIB and IId/x MyHC genes show the highest homology, although complete sequence for these three genes is not available to allow a full comparison. Over a segment of 613 amino acids, human MyHC-IIB and IId/x show 94% identity (Weiss and Leinwand, 1996). IId/x and IIB gene transcripts can be detected between days 2 and 5 of post-natal life in the mouse and are coexpressed in many muscle groups including masseter, tongue, quadriceps, plantaris, soleus, psoas, gastrocnemius, tibialis anterior, and extensor digitorum longus (EDL). The most prominent MyHC in the adult skeletal musculature is IIB where it comprises >70% of the myosin overall. MyHC-IId is less abundantly expressed but comprises 30–60% of the MyHC in diaphragm, tongue, masseter, plantaris, and tibialis anterior (Jansen et al., 1996).

To determine whether the molecular diversity in the vertebrate myosin heavy chain gene family represents functional diversity, we initiated an effort to generate strains of mice carrying mutations in different MyHC genes. Here we report the successful targeted inactivation of the adult fast skeletal myosin heavy chain genes MyHC-IIB and IId/x in embryonic stem cells and derivation of mice carrying the mutation. Mice which are null for the MyHC-IIB and IId/x gene are viable and fertile, but weigh less than wild type by 10 wk of age, and this lower weight is maintained. At young ages (4–6 wk), IIB null mice exhibit gross limb muscle weakness which resolves with age, whereas IId/x null mice have chronic limb weakness. Distinct physiological defects were seen in muscles from both null strains. IIB

null mice are severely impaired in the amount of force generated/cross sectional area of muscle, whereas IId/x null mice have normal force generation, but delayed kinetic properties. A large proportion of homozygous IId/x null mice and a small number of heterozygotes develop severe curvature of the spine (kyphosis). Distinct histological abnormalities were found in both null strains. These results suggest that MyHC-IId/x and IIB are required for the normal function of adult skeletal muscle and that mice missing these gene products display distinct phenotypes.

Materials and Methods

Generation of MyHC^{-/-}-deficient Mice

Clone E14-1 ES cells were cultured according to Robertson (Robertson, 1987) except that the medium was supplemented with leukemia inhibitory factor (GIBCO BRL, Gaithersburg, MD) at 1,000 U/ml. Approximately 2×10^7 E14-1 ES (Fodde, 1994) cells were resuspended in electroporation buffer (10 mM HEPES-PBS, pH 7.05). Cells were mixed with 100 μ g of the linearized targeting constructs and subjected to a 4.1-ms pulse at 400 V, 250 uFA. Cells were divided equally onto 10 10-cm dishes containing feeder cells and ES-cell medium and grown for 36 h before addition of 150 μ g/ml G418 (Geneticin; GIBCO BRL) for positive selection. Cells were cultured for 7–10 d, until such time as discrete G418 resistant colonies appeared on the plates. 140 individual colonies were picked, trypsinized, and transferred to individual wells of 24-well dishes containing ES-cell medium and feeder cells. DNA from individual clones was analyzed by Southern blot hybridization with probes flanking the construct integration site.

Clones in which homologous recombination had taken place were injected into C57BL/6 recipient blastocysts which were transferred to CD-1 pseudopregnant females (Robertson, 1987). Genotyping of mice was done by Southern analysis as described above.

Analysis of RNA

A 90-bp PCR fragment containing the 3'UTR of the MyHC-IId/x gene was generated from genomic mouse DNA using a specific primer pair generated based on published mouse sequences. They are RK-1509 (5'-GCCATGTTAATGTGGAGACC-3') and RK-1510 (5'-CATGCTTCCTACCTTCTC-3'). Similarly, the probe for the MyHC-IIB gene was constructed using a 120-bp PCR fragment corresponding to the 3'UTR. The primer pair used to amplify this fragment from mouse genomic DNA are IIB3F (5'-ACAAGCTGCGGGTGAAGAGC-3') and IIB3R (5'-CAGGACAGTGACAAAGAACG-3'). The product was blunt-ended using T4 Polymerase (GIBCO BRL), and ligated into EcoRV digested Bluescript SK⁺/– cloning vector (Stratagene Inc., La Jolla, CA) thus placing them under the control of the T7 and T3 promoters. The transcriptional orientation of the insert in each probe was assessed by sequencing individual clones.

The transcription reaction was performed with the Maxiscript kit (Ambion Inc., Austin, TX) according to the manufacturer's protocols. The mouse pTRI-actin template used to make the 360-bp mouse β -actin antisense probe was provided with the kit. RNA transcript of high specific activity was generated either by digesting the plasmid with XbaI and transcribing with the T7 polymerase enzyme in the presence of [³²P]UTP, or by cutting the plasmid with XhoI and transcribing with the T3 polymerase enzyme in the presence of [³²P]UTP. Ribonuclease protection assays were performed with the RPA II kit (Ambion) according to the manufacturer's protocol.

Myofibrils, Western Blot, and High Resolution Electrophoresis

Myofibrils were prepared from the tibialis anterior muscles of 5-mo-old female mice. Muscles were minced with scissors and essentially prepared as described by Solaro et al. (1971). Tissues were homogenized in 1 ml of buffer A (50 mM KCl, 10 mM KPO₄, 2 mM MgCl₂, 0.5 mM EDTA, 2 mM DTT, pH 7.0). The homogenate was separated by centrifugation at 15,800 g for 15 min at 4°C. The resulting pellets were resuspended in 1 ml of buffer C (buffer A + 1% Triton X-100) and again separated by centrifugation (15,800 g for 15 min at 4°C). Pellets were resuspended in 1 ml of buffer (60

mM KCl, 30 mM imidazole, 2 mM MgCl₂, 1 mM DTT, pH 7.0) and stored at -70°C. 5 µg of total protein was either separated on a 10% SDS-polyacrylamide gel or blotted onto nitrocellulose (Bio-Dot; Bio Rad Labs., Hercules, CA). The gel was stained with Coomassie blue and the nitrocellulose was probed with an antibody specific to MyHC-IIb (BFF3) (Schiaffino et al., 1986). High resolution gel electrophoresis was performed according to Talmadge and Roy (1993) after homogenization of tongue and preparation of myofibrils as described above.

Grip Strength Measurement

Muscle strength in mice was measured using an automated Grip Strength Meter (Columbus Instruments, Columbus, OH). The instrument is designed to measure both fore- and hindlimb grip strength in small laboratory rodents. The instrument uses an electronic digital force gauge which measures the peak force exerted upon it by the action of the animal.

For the forelimb grip strength measurement, the animal was held by the base of the tail and allowed to place its forepaws on the flat wire mesh of the pull bar connected to the force gauge. The animal was slowly pulled away from the pull bar at a rate of ~1 inch/s until it released the pull bar. Peak tension was recorded from the force gauge's digital readout. The hindlimb grip strength measurement was performed in a similar manner, but allowing the animal to grasp the pull bar with both front and hind paws.

Metabolic Studies

Two separate metabolic cages (Nalge Co., Rochester, NY) were used to group-house five wild-type mice and five homozygous null *IId/x* mice at 4 wk of age. 4 d were allowed for acclimatization; for an additional 7 d, food and water intake were followed in these two groups of mice.

X-Ray Analysis

One wild-type male mouse (12 wk old) and one MyHC-IIb^{-/-} male mouse which displayed visible kyphosis (8 wk old) were chosen for x-ray analysis. The mice were anesthetized and x-rayed by a veterinarian.

Muscle Physiology

Experimental Apparatus. The experimental chamber consisted of a plastic trough with a glass bottom filled with Ca²⁺ Krebs solution. Solutions in the chamber were continuously bubbled with oxygen and kept at 22°C as described previously (Metzger et al., 1985). Muscles were mounted in the chamber between a force transducer (model 400; Cambridge Technology, Inc., Watertown, MA) and a moving coil galvanometer (Cambridge Technology, Inc.) was used to rapidly release the muscle. Both the force transducer and the galvanometer were mounted on three-way positioners to allow the muscle strips to be properly stretched and aligned. Muscle strips were electrically stimulated through the use of platinum electrodes glued to the sides of the trough.

The EDL was used to assess changes in the mechanical response of the myosin IIb^{-/-} mouse. Mice were anesthetized by injection with nembutal. The tendons of the EDL in one leg were exposed and tied with 4-0 suture. The muscle was then isolated, the tendons cut, and the muscle removed from the animal and placed in Ca²⁺ Krebs solution on ice. The Krebs solution was continuously bubbled with oxygen. The EDL from the other leg was then removed in a similar manner. The EDL was mounted in the experimental chamber between the force transducer and a moving coil galvanometer by means of hooks pushed through the tendons at the level of the ties. The fiber length and the stimulation voltage were then adjusted to produce a maximum twitch force response, and the stimulation frequency necessary to produce a fused tetanus determined. At the end of the experiment the length of their muscle was measured in the experimental chamber. The muscle was then cut out and stored in SDS buffer for later analysis of the protein composition. Cross-sectional area (CSA) was determined by dividing the mass by the length and density of the muscle. Muscle density was assumed to be 1.06 mg/mm³ (Hill, 1931).

Diaphragm Muscle Strips. Mice were anaesthetized by injection with Nembutal and the diaphragm, along with the adjoining portion of the rib cage, was removed. The diaphragm was immediately placed in a small Petri dish filled with Ca²⁺ Krebs on ice. The solution was continuously bubbled with oxygen. The diaphragm was then cut into 6-8 strips ~0.5-0.1 mm in width with a piece of the central tendon and the adjoining rib intact. These muscle strips were slightly stretched, pinned down, and kept on ice until transfer to the experimental chamber. Holes were pushed

through the central tendon and the rib to allow the muscle strip to be mounted in the experimental chamber on hooks attached to the force transducer and galvanometer. The optimum length and stimulus voltage were then determined by monitoring the twitch force response, and the dimensions of the muscle strip determined. After the experiment, the diaphragm strip was removed from the chamber, the tendons carefully removed and the strip weighed. A small segment from the center of the bundle was then placed in SDS buffer and stored for later analysis of the protein composition. CSA was determined by dividing the mass by the length and density of the muscle strip. Muscle density was assumed to be 1.06 mg/mm³ (Hill, 1931).

Histology

Muscle tissues were isolated from male mice at 4-6 wk of age and rapidly frozen in liquid nitrogen cooled isopentane. 10-µm sections were cut on a Tissue-Tek cryostat and mounted on poly-lysine-coated cover-slips. Hematoxylin and eosin, Masson trichrome, and NADH-tetrazolium reductase (NADH-TR) staining were performed according to standard procedures.

Results

Targeted Disruption of MyHC-IIb and MyHC-IIId/x in the Mouse

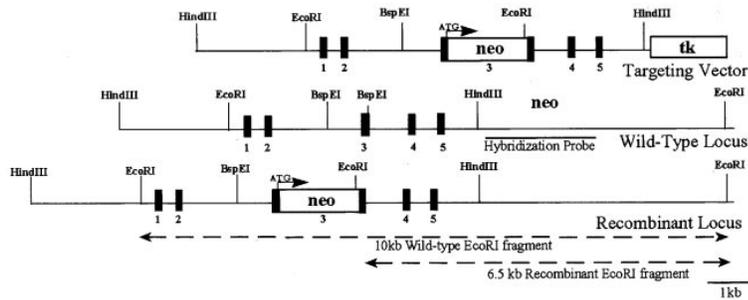
A 2.6-kb probe consisting of the 5' half of a full-length human perinatal MyHC cDNA clone (Karsch-Mizrachi et al., 1990) was used to screen a mouse genomic library (129/Ola, gift from Dr. O. Smithies, University of North Carolina, Chapel Hill, NC). One of the resulting clones, λ19a, contained sequences identical to the 3'-end of the previously characterized MyHC-IIa gene (Parker-Thornburg et al., 1992); this gene is known to lie 4 kb upstream of the MyHC-IIId/x gene whose first five exons were also present on λ19a. A second clone, λ10a was found to have 99% identity over a stretch of 383 bp to noncoding sequence (data not shown) previously identified in an isolated cosmid clone as the promoter region of the adult mouse MyHC-IIb gene (Takeda et al., 1992).

The MyHC-IIb targeting vector (Fig. 1 A) was constructed from a genomic fragment which contained part of the 5' end of the MyHC-IIb gene, including the first two non-coding (exons 1 and 2), and the first three coding exons (exons 3-5). A 1.8-kb fragment containing the PGK-Neo cassette was inserted into a BspEI site in the third exon of the MyHC-IIb gene. The transcriptional orientation of the Neo cassette was opposite to that of the MyHC gene. A PGK-TK cassette was inserted downstream of the modified gene for negative selection.

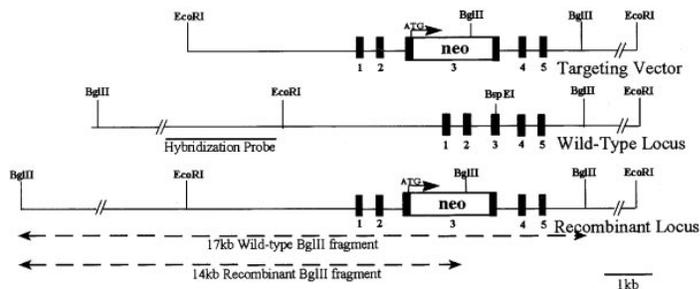
The MyHC-IIId/x targeting vector (Fig. 1 B) was constructed from an 11-kb genomic fragment which contained the 5' end of the MyHC-IIId/x gene including its first two noncoding exons and the first three coding exons. This fragment contains a unique BspEI site which maps to the third exon of the *IId/x* gene. A 1.8-kb fragment containing the PGK-Neo cassette was inserted into the BspEI site. The transcriptional orientation of the Neo cassette was the same as that of the MyHC gene.

The targeting constructs were introduced into E14-1 ES cells by electroporation and individual G418 resistant clones were isolated and genotyped (Hooper et al., 1987). 144 drug-resistant clones were screened for targeted disruption of MyHC-IIb using a 2.5-kb EcoRV/HindIII genomic fragment not included in the targeting vector. This probe

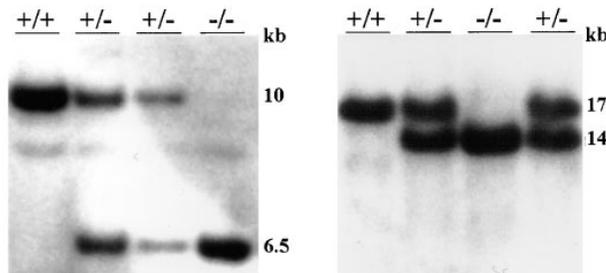
A Targeting Strategy: Myosin Heavy Chain IIb



B Targeting Strategy: Myosin Heavy Chain IId/x



C



detects a 10-kb EcoRI fragment in wild-type cells and 6.5-kb fragment from properly targeted ES cells. 2 of 144 clones analyzed were found to have the appropriately modified locus.

180 drug-resistant clones were screened for targeted disruption of MyHC-IId/x using a 1.6-kb EcoRI genomic fragment not included in the targeting vector. This probe detects a 17-kb BglII fragment in wild-type cells and 14-kb fragment from properly targeted ES cells. 42 of 180 (23%) of the clones analyzed were found to have the appropriately modified locus.

Two clones (F2b c.4 and c.7) heterozygous for the MyHC-IIb mutation were injected into C57Bl/6J blastocysts. Chimeric male animals derived from the F2b c.7 ES cell clone transmitted the mutation to their offspring when bred to C57Bl/6J females. F1 heterozygous animals were interbred. The resulting offspring were genotyped by Southern blot analysis using the 2.5-kb EcoRV/HindIII fragment of the probe. Representative results are shown in the first panel of Fig. 1 C. Of the 59 mice examined, 12 were IId/x+/+, 29 were IId/x+/-, and 17 were IId/x-/-.

Figure 1. The MyHC-IIb and IId/x targeting strategies and Southern blot analysis of F2 mice. Targeting vector, wild-type and recombinant loci are shown with relevant restriction sites. (A) A 6-kb HindIII fragment containing the first five exons of the MyHC-IIb gene was subcloned from the λ 10a clone. The PGK-neomycin cassette was inserted into the third exon, downstream of the translational start site, and in the opposite orientation to the MyHC gene. The PGK-thymidine kinase gene for negative selection was placed 3' of the construct as shown. Homologously recombined clones were assayed for by EcoRI Southern blot analysis using a probe 3' of the construct, as indicated. (B) A 11-kb EcoRI fragment containing the first five exons of the MyHC-IId/x gene was subcloned from the λ 19a clone. The PGK-neomycin cassette was inserted into a unique BspEI site, downstream of the translational start site, in the same orientation as the MyHC gene. Homologous recombination was assayed by BglII Southern Blot analysis using a probe 5' of the targeting construct, as shown. (C) The first panel depicts a EcoRI southern blot analysis of F2 mice from the IId targeting experiment. Wild-type, heterozygous and homozygous null animals are shown. The second panel depicts a BglII southern blot analysis of F2 mice from the IId/x targeting experiment.

Four clones heterozygous for the MyHC-IId/x mutation were injected into C57Bl/6J (B6) blastocysts. Chimeric male animals derived from three independent ES cell clones transmitted the mutation to their offspring when bred to B6 females. F1 heterozygous animals were interbred. The resulting offspring were genotyped by Southern blot analysis using the 1.6-kb EcoRI fragment as the probe. Representative results are shown in the second panel of Fig. 1 C. Of the 114 mice examined, 22 were IId/x+/+, 55 were IId/x+/-, and 37 were IId/x-/- . These results indicate that the modified loci are transmitted in a Mendelian fashion and the modifications do not appear to affect normal prenatal development.

Analysis of MyHC Gene Expression in Null Mice

We used two different methods to determine the effect of the genetic modification on MyHC-IIb and IId/x gene expression. RNase protection was chosen for the analysis of MyHC mRNA expression levels. RNA samples from five different mouse muscle tissues (heart, diaphragm, whole

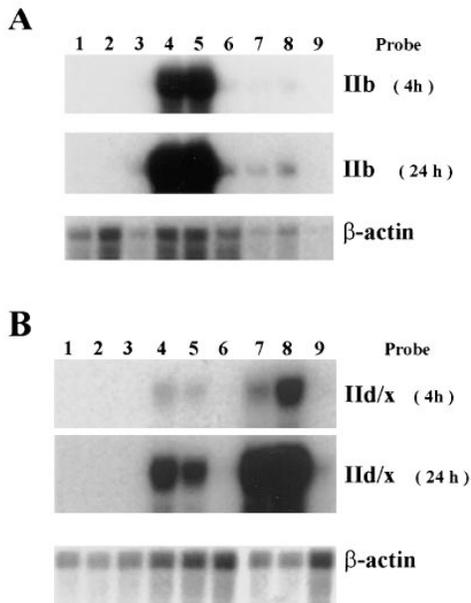


Figure 2. RNA analysis. In both the Iib and Iid/x animals, total RNA from heart (lanes 1–3), lower leg (lanes 4–6), and masseter (lanes 7–9) was examined for the expression of Iib and Iid/x RNA, respectively. A β -actin probe was used to control for the amount of RNA. (A) The expression of MyHC-Iib was analyzed by RNase protection using a 120-nt fragment containing the 3' UTR for the MyHC-Iib gene. The heart RNA is negative for Iib expression in wild-type (lane 1), heterozygous (lane 2), and homozygous null animals (lane 3). In skeletal muscle samples, wild-type (lanes 4 and 7) and heterozygous (lanes 5 and 8) but not homozygous animals (lanes 6 and 9) express of Iib RNA. (B) The expression of MyHC-Iid/x RNA was analyzed using a 90-nt fragment containing the 3' UTR for the gene. The heart RNA is negative for expression in wild-type (lane 1), heterozygous (lane 2), and homozygous null animals (lane 3). The lower leg and masseter samples show expression in wild-type (lanes 4 and 7), and heterozygous animals (lanes 5 and 8), but not in homozygous null samples (lanes 6 and 9).

lower leg, masseter, and tongue) were studied using this method. Representative results are shown in Fig. 2 of RNase protection analysis of mRNA from masseter and lower-leg tissue using a probe specific to the 3'-UTRs of the two MyHC genes. These results show that while $+/+$ and $+/-$ animals express the MyHC-Iib and Iid/x genes, MyHC-Iib and Iid/x $-/-$ animals had little or no detectable MyHC-Iib or Iid/x mRNA expression, respectively. This was true for all five muscle sources tested (data not shown). Analysis of the same samples with a β -actin probe revealed approximately equal expression levels of these mRNAs in samples from all three groups of animals (Fig. 2). To confirm that the homologous recombination event resulted in a null allele for protein expression, myofibrils were purified from tibialis anterior, which in $+/+$ mice expresses high levels of MyHC-Iid/x and Iib. To confirm that the Iib mice are null for Iib protein, immunoblot analysis was carried out with a mAb specific for MyHC-Iib. Fig. 3 A shows myofibrils from the tibialis anterior muscles of Iib mice separated by SDS-PAGE. The same amounts of protein as in Fig. 3 A (lanes 2–4) were blotted onto nitrocellulose and probed with an antibody specific to MyHC-Iib (Fig. 3 B).

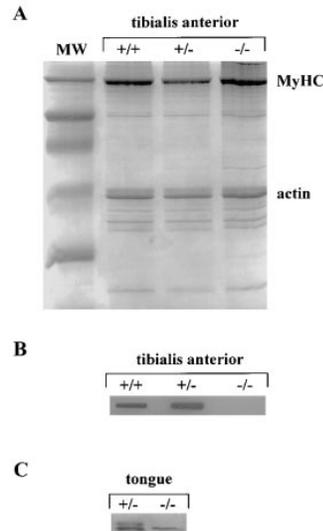


Figure 3. MyHC-Iib protein expression. Myofibrils were prepared from tibialis anterior muscles of MyHC-Iib $+/+$, MyHC-Iib $+/-$, and MyHC-Iib $-/-$ mice. (A) Myofibrils were separated by SDS-PAGE and stained with Coomassie blue. Lane 1, MW; lane 2, Iib $+/+$; lane 3, Iib $+/-$; lane 4, Iib $-/-$. (B) Myofibrils were blotted onto nitrocellulose and probed with an antibody against MyHC-Iib (BFF3). (C) High resolution gel electrophoresis of MyHC from tongue from $+/+$ and $-/-$ Iid mice.

Whereas Iib $+/+$ and Iib $+/-$ mice contain Iib protein, there is no detectable MyHC-Iib protein in $-/-$ mice. An additional point shown by Fig. 3 A is that the normal actin/myosin ratio appears normal. Because there is no antibody specific for MyHC-Iid/x, high resolution protein gel expression was carried out on myosin from tongue, which in $+/+$ mice, contains MyHC-IIB (faster migrating species) and MyHC-II/x (slower migrating species). As shown in Fig. 3 C, there is no detectable MyHC-Iid/x in $-/-$ mice.

Reduced Whole Body Weight in MyHC Null Mutants

Both MyHC-Iib $-/-$ and MyHC-Iid/x $-/-$ animals were significantly smaller in size than their $+/-$ and $+/+$ littermates by 10 wk of age. wild-type, heterozygous, and homozygous male mice were weighed once a week for a period of 100 d. The data, shown in Fig. 4, reveal that, by 40 d of postnatal life, the $-/-$ mice begin to show consistent growth retardation. By 100 d of age, they have an average weight $\sim 20\%$ less than their $+/+$ and $+/-$ littermates. To determine whether the lower body weight was due to decreased muscle mass, the masses of 8 muscle groups were determined for $-/-$, $+/-$, and $+/+$ animals from the MyHC-Iid/x line at 6 wk of age. Tibial lengths were also determined. These data are shown in Table I. The tibial lengths for all three groups in the Iid/x mice were nearly identical, indicating that the differences in body weight were not due to a bony skeletal developmental defect, or retardation in bone growth. Three of the eight muscle groups had significantly lower muscle mass than the $+/-$ mice; however, no quantitative correlation could be made between the decreased muscle mass and the reported Iid/x MyHC content of wild-type muscle. In the case of the Iib mice, there was also nonuniform decrease in muscle mass. However, different muscles relative to the Iid/x $-/-$ were affected and to a greater degree than in Iid $-/-$ mice. For example, gastrocnemius and vastus lateralis in Iib $-/-$ mice were both $< +/-$ the mass of $+/+$, while the tibialis anterior was of normal mass (not shown). This is despite the fact that all three muscles have high MyHC-Iib content in the wild type mouse.

Based on the high expression levels of Iid/x in masseter, tongue and diaphragm in wild-type animals, it seemed pos-

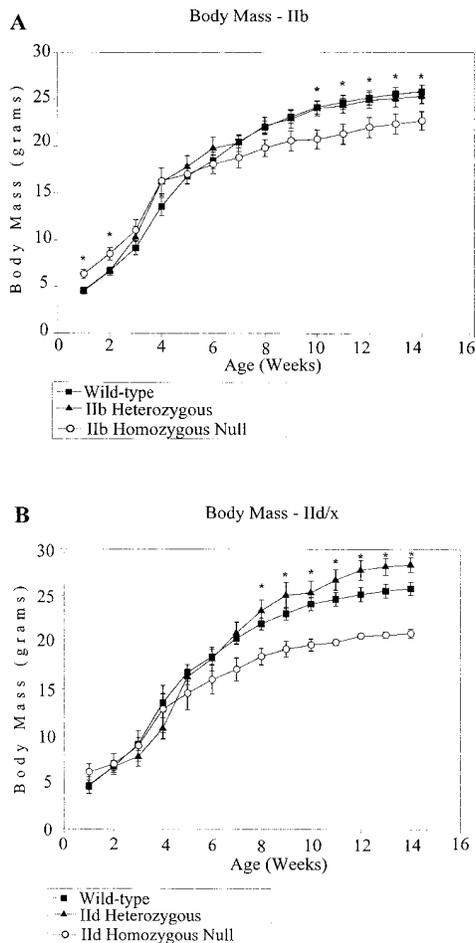


Figure 4. Growth rates of MyHC-IIb and IId/x mice. Weight measurements for wild-type (■), heterozygous (▲), and homozygous (○) male animals were taken once a week for a time period of 100 d. The homozygous null mice weigh significantly less than their +/+ or +/- littermates. Asterisks indicate time points at which a statistically significant difference ($P < 0.05$) exists between combined average +/+ and -/- body weights. For the IIb mice, $n = 14, 4,$ and 4 ; for the IId/x mice, $n = 14, 7,$ and 8 for wild-type, heterozygous, and homozygous mice, respectively.

sible that the weight loss observed in these animals may be related to problems the animals may experience with breathing and eating. To address the possibility that smaller mass in these animals may, in part, be a consequence of malnutrition, studies geared to detect possible anomalies in food and water intake in these animals were initiated. Over a course of 7 d, wild-type male mice consumed an average of 16.3 ± 1.6 gm of dry food per day ($n = 5$), while homozygous null IId/x mice consumed 14.0 ± 2.0 gm of food per day ($n = 5$), or $\sim 13\%$ less than wild-type ($P < 0.05$). Similarly, the wild-type cohort consumed an average of 28.7 ± 1.5 ml of water per day while homozygotes consumed 20 ± 2.8 gm of water per day, or 30% less than wild-type ($P < 0.001$).

Muscle Weakness in MyHC-IId/x and IIb Mutants

Based on an empirically assessed observation of muscle weakness in both MyHC null mice at 4–6 wk of age, a study of the comparative fore- and hindlimb grip strengths

Table I. Muscle Mass and Tibial Length Measurements from 6-wk-old Male Mice

Genotype	IId +/+	IId +/-	IId -/-
Number Studied	3	4	6
Gastrocnemius (mg)	215.1 ± 33.9	201 ± 23.2	146.0 ± 28.9
Soleus	15.9 ± 3.6	19.13 ± 6.50	11.70 ± 1.0
Tibialis anterior	55.6 ± 5.8	59.78 ± 2.1	$44.9 \pm 3.8^*$
EDL	12.1 ± 1.1	15.58 ± 0.6	$9.6 \pm 1.3^*$
Psoas	81.7 ± 1.6	74.95 ± 15.8	57.0 ± 3.5
Diaphragm	78.1 ± 6.7	87.6 ± 6.9	72.2 ± 3.4
Masseter	152.7 ± 12.0	146.6 ± 8.4	$100.7 \pm 6.5^*$
Tongue	57.5 ± 11.8	73.3 ± 11.9	54.7 ± 5.10
Body weight (g)	23.4 ± 0.5	24.13 ± 0.9	$17.0 \pm 1.1^*$
Tibial Length (mm)	16.3 ± 0.10	16.42 ± 0.1	16.2 ± 0.1

* $P < 0.01$ vs. +/- or +/+

of MyHC-IIb and IId/x mice was conducted. Five peak grip strength measurements for each mouse for fore- and hindlimbs were performed on +/+, +/-, and -/- mice at 6 wk of age, and at 6 mo of age. Fig. 5 shows the hindlimb measurements for wild-type and null animals at both time points. Heterozygotes were indistinguishable from wild-type (not shown). MyHC-IIb null mice showed decreased grip strength in both sets of limbs at 6 wk of age, but by 6 mo of age, they were indistinguishable from wild type. At 6 wk, this difference was 20% for forelimb strength and 30% for hindlimb strength. For the MyHC-IId/x line, mice

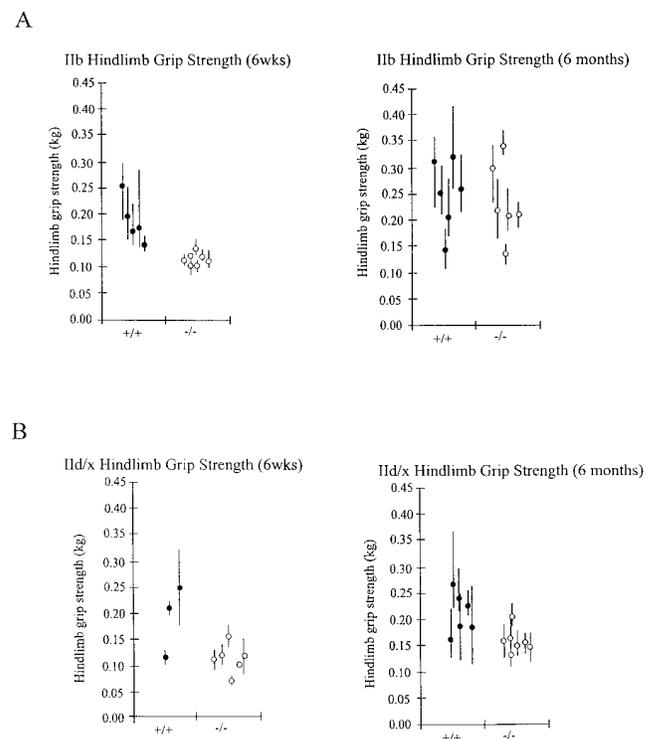
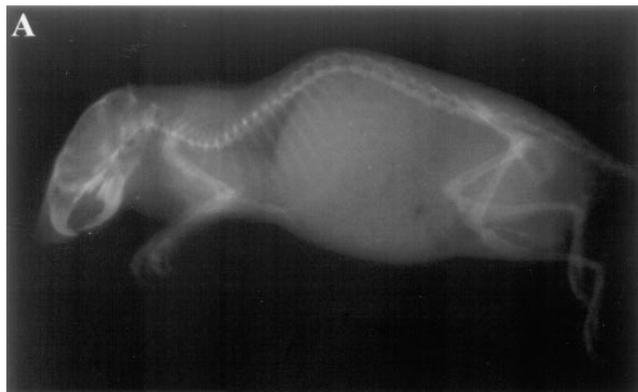


Figure 5. Hindlimb grip strength measurements in MyHC-IIb and IId/x mice. Peak grip strengths were measured on the hindlimbs of mice. Five individual measurements were made for each mouse. Each point in the graph represents the average of the five measurements. There is a statistically significant difference ($P < 0.01$) between the combined average grip-strengths between -/- and +/+ mice at 6 wk of age, but at 6 mo, only the IId/x null mice exhibit sustained deficiencies in grip strength.



Male IId/x Wild-type



Male IId/x Homozygous Null (-/-)

Figure 6. Kyphosis in MyHC-IId/x null mice. Male mice at 12 wk of age (wild-type) and 8 wk of age (MyHC-IId/x^{-/-}) were anesthetized and x-rayed. (A) MyHC-IId/x wild-type ^{+/+} male. (B) MyHC-IId/x^{-/-} male.

of both age groups showed significant sustained muscle weakness. At 6 wk, this difference was 25% for forelimb strength and 40% for hindlimb strength. This difference was maintained with age.

Kyphosis in MyHC-IId/x Mutants

Approximately one-third (8/26) of IId/x^{-/-} knockout mice (males and females) developed kyphosis, a curvature of the spinal column that is commonly referred to as “hunchback.” This deformity is located in the thoracic vertebrae, and presented by as early as 6 wk of age and has been occasionally seen in heterozygous animals. No wild-type littermates developed this phenotype. This phenotype was detectable by visual examination (not shown) or by x-ray (Fig. 6).

Histologic Analysis of Muscles from MyHC^{-/-} Mutants

Tibialis anterior muscle was isolated from mice 4–6 wk of age, and analyzed histologically. This muscle was chosen because it expresses relatively high levels of both MyHC-IId and IId/x and therefore would allow us to compare histological changes between the two null strains (Pette and

Staron, 1990). The results of this experiment are shown in Fig. 7, where sections were stained with hematoxylin and eosin, Masson trichrome and NADH-TR.

There is a notable difference in fiber size between sections of wild-type (Fig. 7, A–C) mice and homozygous null mice (Fig. 7, D–I). It is also apparent that the histological phenotypes of the two null strains are quite different from each other. The mean fiber size increases dramatically in the IId^{-/-} mice. The sizes in wild-type mice range from 1,200 μm^2 (fast glycolytic) to 2,500 μm^2 (fast oxidative). In IId^{-/-} mice, the mean fiber size is substantially increased to >4,000 μm^2 and the fibers are generally uniform in size. In the case of the IId/x null mice, there was a major shift in the geometry and size of the fibers. There was an increase in the number of very small (1–500 μm^2) and large (>2,500 μm^2) fibers relative to the ^{+/-} and ^{+/+} mice. For example, ~38% of the fibers in the tibialis anterior of IId/x^{-/-} mice were <500 μm^2 , compared with 11 and 16% in ^{+/-} and ^{+/+} mice, respectively. The changes in fiber diameter in both lines of mice are statistically significant. Muscles from MyHC-IId and IId/x^{-/-} mice show increased interstitial fibrosis compared with muscles from wild-type mice (Fig. 7, E and H). In both null strains, there is increased spacing between fibers, but it is more notable in the IId null mice. Fibers from IId/x^{-/-} mice were ultrastructurally abnormal with a disorganized appearance of the intermyofibrillar network which can be visualized on NADH-TR stained section (Fig. 7 I). Contrast this phenotype with that seen in NADH-TR staining of the IId^{-/-} section (Fig. 7 F), where the staining is much more uniform when compared with either ^{+/+} (Fig. 7 C) or IId/x^{-/-} (Fig. 7 I). The masseter and psoas also showed histological abnormalities, including exaggerated interstitial fibrosis characterized by a dramatic increase in the number of nuclei in the interstitium. We did not see any indication of central nuclei, which would be an indication of regeneration.

Physiologic Analysis of Muscle from MyHC Mutants

The reduced grip strength found in null mice could be explained by an alteration of contractile function in the striated muscles from these animals. To directly test whether maximum force generating capacity is affected in ^{-/-} animals, we examined peak isometric tetanic tension in EDL and diaphragm muscle strips isolated from 5–7-mo-old IId and IId/x^{-/-} animals, respectively. These muscles were selected for study because of their significant content of IId and IId/x myosin, respectively in wild-type mice (Jansen et al., 1996). Results of this analysis are summarized in Table II and graphed in Fig. 8.

The maximum tetanic force produced by EDL from homozygous mutant IId mice was significantly reduced. This reduction is due to two factors. First, CSA is reduced from $0.83 \pm 0.06 \text{ mm}^2$ (mean \pm SEM, $n = 5$) obtained in control animals to $0.44 \pm 0.03 \text{ mm}^2$ ($n = 6$) homozygous mutant mice. Second, the force/CSA (F/CSA) is significantly reduced from the control value of $262.8 \pm 20.6 \text{ kN/m}^2$ ($n = 5$) to a value of $138.2 \pm 27.9 \text{ kilonewton/m}^2$ ($n = 6$) in homozygous mutant animals. EDL from heterozygous mutant animals gave results intermediate between the control and homozygous mutants. Thus, the absence of type IId fibers has significant effects on the ability of EDL to gener-

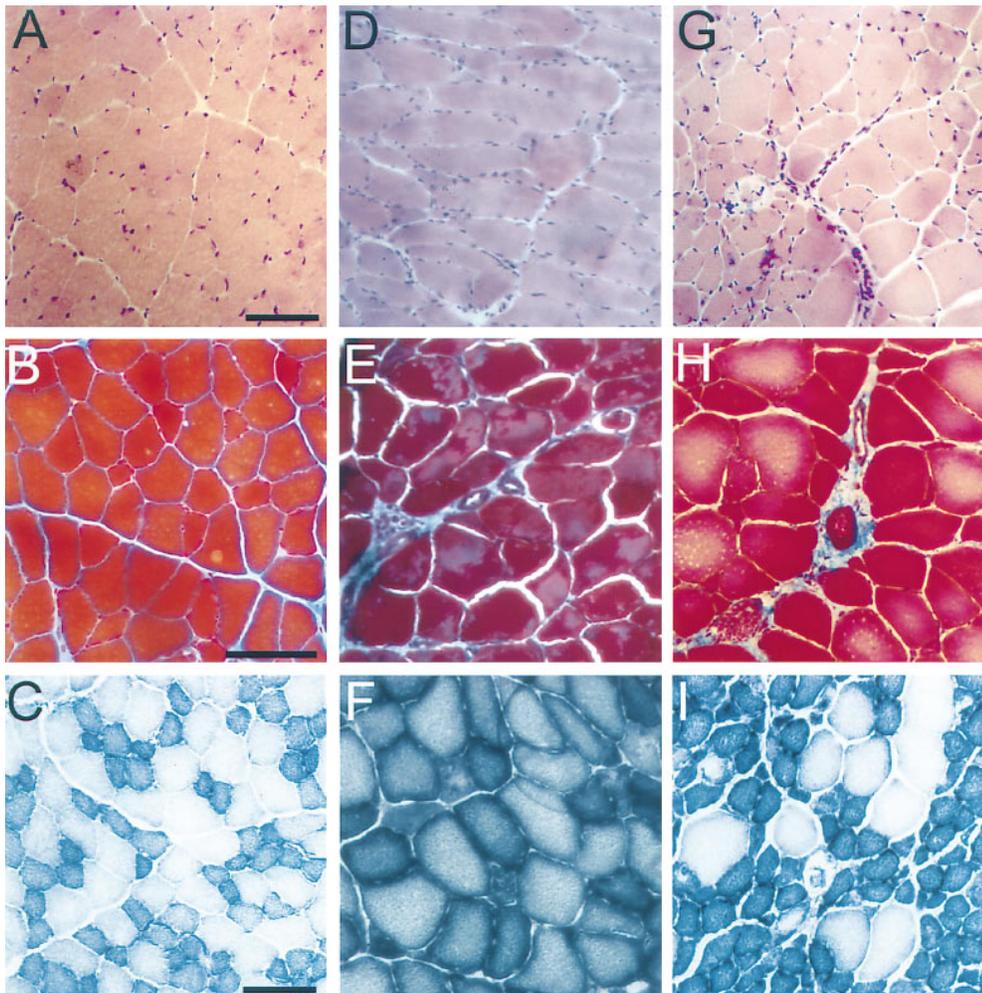


Figure 7. Histologic analysis of tibialis anterior muscle from MyHC-IIId/x mice. Cross-sections of tibialis anterior muscle from 6-wk-old wild-type (A–C), MyHC-IIb^{-/-} (D–F), and MyHC-IIId/x^{-/-} (G–I) mice were stained with hematoxylin and eosin (H&E) (A, D, and G), Masson trichrome (B, E, and H), and NADH-TR (C, F, and I). Note the general disarray (MyHC-IIId/x^{-/-}, G), change in fiber diameter in H&E and NADH-TR stains (F and I) and the interstitial fibrosis visualized with Masson Trichrome staining (E and H).

ate force independently of muscle size. Whether this difference is manifested by a reduction in the percentage of the CSA occupied by functional muscle fibers or a lower level of force produced by those fibers which are present cannot be determined from these data.

Diaphragm muscle strips from homozygous IId/x null mutants displayed wild-type maximum normalized force. The values of F/CSA of diaphragm muscle obtained in this study are somewhat lower than those reported previously by us and other investigators using rodent diaphragm muscle strips (Metzger et al., 1985). This difference likely arises from the smaller width diaphragm strips used in the present study (1 mm) which was done to assure the parallel alignment of muscle fibers within the isolated muscle strips. Despite normal force generation, there were significant alterations in the time-course and kinetics of force development in IId/x^{-/-} diaphragm. Specifically, the time to peak tension was significantly longer as was the time to relaxation (Table 2). In addition, the peak rate of rise of tetanic tension and rate constant for relaxation were significantly reduced in the diaphragm muscle strips from IId/x^{-/-} as compared with IId/x^{+/+} mice.

Discussion

To examine the function of the adult fast skeletal myosin

heavy chain isoforms in normal muscle development and function, we generated MyHC-IIb^{-/-} and MyHC-IIId/x^{-/-} mice. Survival appeared not to be affected in either strain, although we have not examined mice beyond ~14 mo of age. Common and distinct features of the two strains are summarized in Table III. Common features include normal fertility and viability, lower body mass, and decreased limb strength in young animals. Distinct features include kyphosis and sustained grip strength deficits in only IId/x null mice. Both null strains had distinct physiological and histological abnormalities. These phenotypes are not ones that could have been predicted based on the abundance and distribution of these two motor proteins.

MyHC^{-/-} Mice Have Reduced Growth Rates and Adult Body Weights

Skeletal muscle typically accounts for ~40–50% of the body-weight of a mammal (Berne and Levy, 1996) and myosin protein accounts for ~40% of the total muscle protein. In 100-d-old mice, there was a decrease in the average body weight of homozygous IId/x null mice relative to wild-type. When muscle masses were determined from 6-wk-old IId/x null mice (Table I) several, but not all, muscle groups showed 20–30% decreases in mass. The food and water intake of IId/x^{-/-} mice was signifi-

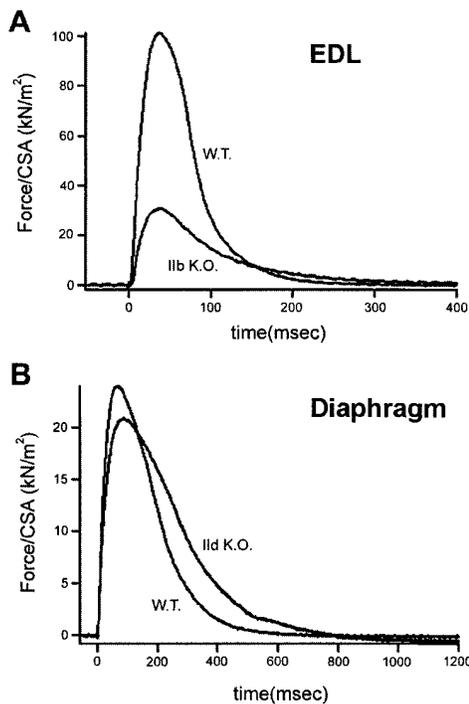


Figure 8. Representative examples of single twitches from whole EDL muscles (A) and small diaphragm strips (B) at 22°C. Muscle length and stimulation voltage (0.5-ms duration) was set to produce maximum twitch force. Muscle was stimulated at time zero. Mouse genotype is as indicated.

cantly less than that of *Iid/x+/+* mice. It seems possible that the MyHC-*Iid* null mice have impaired ability to feed on mouse chow due to reduced function of the jaw muscles. Consistent with that hypothesis is the observation that $-/-$ mice cannot be distinguished by weight from their $+/+$ and $+/-$ littermates until after weaning. Experiments are in progress to determine whether the reduced body weights of the mice can be prevented, in part, by soft food diet. In the case of the *Iib* null mice, the alterations in muscle mass are quite dramatic, but once again, not always correlated with their *Iib* content in wild-type mice. Fur-

Table III. Comparison of MyHC-*Iib* and *Iid/x* Null Mice

	<i>Iib</i>	<i>Iid/x</i>
Viability	WT	WT
Fertility	WT	WT
Grip Strength		
Young animals	↓	↓
Old animals	WT	↓
Body Mass	↓	↓
Kyphosis	-	+
Muscle Mass	±	±
Force Generation/CSA	↓	WT
Time to peak tension	WT	↑
Time to relaxation	WT	↑
Histology		
Fiber diameter	↑	↑ & ↓
Interstitial fibrosis	+	+
Disorganization/degeneration	WT	+

ther metabolic studies are required to assess this phenotype in both null strains; but it is quite clear that this is a complex phenotype that may result from structural problems of the muscle as well as secondary and tertiary sequelae.

Possible Basis of the Kyphosis

There is a clear link between spinal curvature and abnormalities in both para-spinal and non-spine-related musculature. A number of human neuromuscular diseases have been described with kypho-scoliotic phenotypes; these include diseases such as spinal muscular atrophy, limb-girdle muscular dystrophy, myotonic dystrophy, and Duchenne muscular dystrophy, to name only four of more than a dozen muscular diseases that often result in spine-deforming complications (Carter et al., 1995; Johnson et al., 1995; McDonald et al., 1995). Thus, many distinct primary defects can result in kyphosis. An autosomal recessive mutation in the mouse, *ky*, causes kyphoscoliosis (Bridges et al., 1992). Muscle in *ky* mice appears to be necrotic and regenerating. EDL muscle from these mice shows a significant defect in force generation. Despite some similarities with the MyHC-*Iid* null strain reported here, there is no apparent defect in MyHC composition and this disease is thought

Table II. Summary of Physiologic Studies

	EDL		Diaphragm	
	<i>Iib</i> +/+	<i>Iib</i> -/-	<i>Iid</i> +/+	<i>Iid</i> -/-
Twitch properties				
CT (ms)	34.2 ± 1.8(6)	38.0 ± 3.2(7)	61.9 ± 4.9(8)	80.7 ± 5.7(8)*
RT _{1/2} (ms)	47.4 ± 5.2(6)	69.6 ± 9.8(7)*	103.6 ± 9.9(8)	154.4 ± 15.5(8)*
P _t (kN.m ²)	91.4 ± 11.5(5)	40.9 ± 6.7(6)*	15.4 ± 3.5(7)	23.1 ± 6.7(8)
Tetanic properties				
P ₀ (kN.m ²)	262.8 ± 20.6(5)	138.2 ± 27.9(6)*	42.6 ± 9.6(7)	53.4 ± 11.4(8)
Fusion frequency (Hz)	76.7 ± 2.1(6)	70.0 ± 3.1(7)	58.8 ± 2.3(8)	58.8 ± 4.0(8)
Tw/Tet	0.30 ± 0.02(6)	0.31 ± 0.11(7)	0.35 ± 0.04(8)	0.39 ± 0.06(8)
+dP/dt* 1/P _{max}	5.45 ± 0.24(6)	5.32 ± 0.58(7)	5.2 ± 0.7(7)	3.4 ± 0.3(8)*
-dP/dt* 1/P _{max} (ms ⁻¹)	7.98 ± 0.38(6)	4.76 ± 0.46(7)*	2.9 ± 0.5(7)	2.0 ± 0.2(8)
K _R (s ⁻¹)	34.4 ± 2.0(6)	24.0 ± 3.5 (7)	14.4 ± 0.3(7)	10.1 ± 1.0(8)*

Values are mean ± SEM (*n*). Asterisks indicate significance at $P < 0.05$. CT is contraction time calculated as time to peak twitch tension, RT_{1/2} is one-half relaxation time, Tw/Tet is twitch to tetanus force ration, K_R is rat constant for relaxation, $+/-$ dP/dt are peak positive and negative rates of change of tension normalized to maximum tetanic force. P₀ is peak tetanic tension and P_t is peak twitch tension, both normalized per cross-sectional area. *Indicates statistical significance of $P < 0.01$.

to be neuromuscular in origin. Kyphosis has also been found in a number of genetically manipulated mice including MyoD null/mdx mice which lack both dystrophin and MyoD (Megeney et al., 1996). In these mice, the paraspinal muscles were shown to be much smaller than in the wild-type animals. Kyphosis was also seen in newborn pups of myogenin $-/-$ mice which do not develop any skeletal muscle (Hasty, 1993). The fact that the psoas muscle is significantly lower in mass in the $-/-$ IId MyHC mice may be an indication that the back musculature is generally not sufficient to hold the spine in place. However, this phenotype was not seen in IIB null mice despite the much greater prevalence of IIB MyHC throughout the skeletal musculature in the wild-type mouse.

Histologic Changes in MyHC $-/-$ Muscle

The histological features of the two different MyHC $-/-$ mice are distinct relative to each other. However, none of the changes seen here are specific to the MyHC gene family since many different human diseases can present with similar phenotypes. For example, increased cellularity and fibrosis in the interstitium, as was seen in this report, have been described in Duchenne muscular dystrophy, limb-girdle dystrophies and neurogenic atrophies.

The disorganization of the intermyofibrillar network seen in the MyHC-IId/x $-/-$ mice is very similar to a clinical syndrome known as "moth-eaten" fibers which, once again, is observed in a number of myopathies. The most striking pathology in IId null mice was seen in the TA with a very large population of small fibers and disorganization of the intermyofibrillar network. It remains to be determined whether this phenotype is the result of muscular atrophy or represents a steady state. In contrast, MyHC-IIB null mice show a dramatic hypertrophy of all fibers in this muscle and do not display substantial disorganization. This phenotypic difference is somewhat surprising given the fact that >90% of the MyHC in this muscle consists of IIB and IId/x protein in wild-type animals.

Muscle Contractility

The normalized peak force generating capacity of the diaphragm is not altered in homozygous MyHC-IId/x mice. This is a somewhat surprising finding considering that IId/x fibers represent ~50% of the fiber type population in normal diaphragm muscles of mice (Jansen et al., 1996). The most likely explanation for this finding is that the IId/x fibers were at least partially rescued by activation of one or several of the other skeletal myosin genes with similar force generating capabilities. If functional compensation is the case, the normalized maximum force would not be expected to change significantly. We have preliminary data which suggest that the MyHC-IIa gene is compensating for the absence of IId/x protein. Examination of potential compensatory changes is obviously a subject for future investigation. While maximum force generating capacity was not changed, we did find alterations in the kinetics of tension generation that tended to slow the contractile behavior of the diaphragm strips from $-/-$ animals. These alterations may have influenced the function of the diaphragm in vivo, and in this case, could underlie, at least in part, some the overall weakness observed in these animals. In

contrast, the EDL muscle, which in wild-type mice has a high MyHC-IIb content, shows a significant decrease in force generated/cross section but no alterations in the kinetic properties of the muscle. Once again, it will be very interesting to determine the properties of individual muscle fibers and their MyHC content.

Implications for human skeletal muscle disease. Studies of the skeletal genes in the context of muscle pathology in *C. elegans* and *D. melanogaster* yielded a wealth of information about their function in the muscle sarcomere and established a clear correlation between MyHC gene mutations and skeletal muscle disease among invertebrates (Dibb et al., 1985; Chun and Falkenthal, 1988). Our study presents evidence for the involvement of a noncardiac MyHC gene in skeletal muscle disease in a higher vertebrate. A number of similarities exist between phenotypes observed in the MyHC mice with those seen in known human congenital myopathies. Muscle weakness and swallowing difficulty from birth along with scoliosis and histologic variation in fiber size and number are phenotypes characteristic of a number of congenital myopathies, including congenital fiber-type disproportion and Escobar syndrome (Yokochi et al., 1985). It would be useful to examine the genetic status of the skeletal MyHC genes in inherited forms of these and other muscle disorders.

We have found that targeted disruption of adult skeletal myosin heavy chain genes results in varying degrees of skeletal muscle related pathology in mice homozygous for these mutations but that the phenotypes are very different and not correlated with the abundance and distribution of the two gene products. This suggests that despite the common origin and high degree of sequence conservation between the members of the adult skeletal MyHC genes, these genes appear to have diverged significantly enough to have acquired distinct and unique roles in skeletal muscle contraction.

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