Inactivation of the myogenic bHLH gene *MRF4* results in up-regulation of myogenin and rib anomalies

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The myogenic basic helix–loop–helix (bHLH) proteins MyoD, myf5, myogenin, and MRF4 can initiate myogenesis when expressed in nonmuscle cells. During embryogenesis, each of the myogenic bHLH genes is expressed in a unique temporospatial pattern within the skeletal muscle lineage, suggesting that they play distinct roles in muscle development. Gene targeting has shown that MyoD and myf5 play partially redundant roles in the genesis of myoblasts, whereas myogenin is required for terminal differentiation. MRF4 is expressed transiently in the somite myotome during embryogenesis and then becomes up-regulated during late fetal development to eventually become the predominant myogenic bHLH factor expressed in adult skeletal muscle. On the basis of its expression pattern, it has been proposed that MRF4 may regulate skeletal muscle maturation and aspects of adult myogenesis. To determine the function of MRF4, we generated mice carrying a homozygous germ-line mutation in the *MRF4* gene. These mice showed only a subtle reduction in expression of a subset of muscle-specific genes but showed a dramatic increase in expression of myogenin, suggesting that it may compensate for the absence of MRF4 and demonstrating that MRF4 is required for the down-regulation of myogenin expression that normally occurs in postnatal skeletal muscle. Paradoxically, MRF4–null mice exhibited multiple rib anomalies, including extensive bifurcations, fusions, and supernumerary processes. These results demonstrate an unanticipated regulatory relationship between myogenin and MRF4 and suggest that MRF4 influences rib outgrowth through an indirect mechanism.

[*Key Words*: Myogenesis; basic helix–loop–helix; muscle gene expression]

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Members of the basic helix–loop–helix (bHLH) class of transcription factors have been shown to specify a variety of embryonic cell fates in vertebrates and invertebrates. A role for bHLH proteins in cell fate specification has been particularly well defined in the neurogenic lineage of *Drosophila*, in which members of the achaete-scute family of bHLH proteins specify the identity of neural cell precursors and subsequently control the formation of specific neural cell types (for review, see Jan and Jan 1993). Defining these types of genetic pathways in vertebrates has been difficult because of the complexity of vertebrate development and because of apparent genetic redundancy. However, recent studies suggest that the formation of vertebrate skeletal muscle may be controlled by a similar type of genetic pathway involving bHLH proteins that act at multiple points in the myogenic lineage.

The myogenic bHLH proteins, MyoD (Davis et al. 1987), myf5 (Braun et al. 1989), myogenin (Edmondson and Olson 1989; Wright et al. 1989), and MRF4/herculin/myf6 (Rhodes and Konieczny 1989; Braun et al. 1990; Miner and Wold 1990), are expressed in vertebrate skeletal muscle, where they appear to constitute a genetic pathway that establishes muscle cell identity and leads to the expression of muscle structural genes during differentiation (for review, see Weintraub 1993; Olson and Klein 1994). When expressed ectopically in nonmuscle cells, each of these factors can activate skeletal muscle genes and can induce one another's expression. These myogenic factors share ~80% amino acid identity with a bHLH region that mediates dimerization and DNA binding to the E-box consensus sequence (CANNTG). E-boxes are present in the control regions of numerous skeletal muscle-specific genes and in many cases have been shown to be essential for expression of those genes (for review, see Emerson 1990; Olson 1990; Weintraub et al. 1991). Although the four myogenic factors dimerize with the same partners and bind the same target sequence with similar affinities, subtle differences in their abilities to activate muscle structural genes have been detected in transfection assays (Yutzey et al. 1990; Chakraborty et al. 1991). In some cases, these differences have been ascribed to the amino and carboxyl termini of these factors, which are divergent (Chakraborty and Olson 1991; Mak et al. 1992).

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Consistent with their postulated roles as determinants of skeletal muscle cell identity, the myogenic bHLH genes are expressed specifically in skeletal muscle and its precursors during embryogenesis. Skeletal muscle in vertebrates is derived from the somites, which form in a rostral-to-caudal progression by segmentation of the paraxial mesoderm lateral to the neural tube (for review, see Buckingham 1992; Wachtler and Christ 1992). Beginning at about day 8.0 postcoitum (p.c.) in the mouse, the rostral somites become compartmentalized to form the dermatome, myotome, and sclerotome, which give rise to the dermis, the axial musculature, and the ribs and vertebrae, respectively. myf5 is expressed in the somite at embryonic day 8.0 (E8), immediately before somite compartmentalization (Ott et al. 1991; Tajbakhsh and Buckingham 1994). Transcripts for myogenin and MyoD appear in the myotome at E8.5 and E10.5, respectively, and transcripts for MRF4 are expressed transiently in the myotome between E9.0 and E11.5 (Sassoon et al. 1989; Bober et al. 1991; Hinterberger et al. 1991; Cheng et al. 1993). Skeletal muscle from the limbs arises from myogenic precursors that migrate into the limb buds from the ventrolateral region of the somite (Chevallier et al. 1977; Christ et al. 1977). myf5 transcripts appear in the limb buds at E11.0, and myogenin and MyoD transcripts are expressed about a half-day later. MRF4 mRNA does not accumulate in the limb musculature until late fetal stages, when it becomes expressed in differentiated muscle fibers; ultimately, it becomes the predominant myogenic factor in adult skeletal muscle.

The myogenic bHLH genes also show unique expression patterns in established skeletal muscle cell lines in tissue culture. Usually MyoD and myf5 are expressed in undifferentiated myoblasts (Davis et al. 1987; Braun et al. 1989), whereas myogenin is up-regulated when myoblasts begin to differentiate in response to depletion of exogenous growth factors (Edmondson and Olson 1989; Wright et al. 1989). MRF4 is not expressed until late in the differentiation program and has been detected in only a few established muscle cell lines (Rhodes and Konieczny 1989; Braun et al. 1990; Miner and Wold 1990). These unique expression patterns suggest that the myogenic bHLH factors may perform distinct functions at different points in the myogenic pathway.

The functions of the myogenic bHLH genes in the embryo have begun to be revealed through gene targeting in transgenic mice. MyoD–null mice are viable and show no obvious skeletal muscle abnormalities (Rudnicki et al. 1992). The only effect of *myoD* inactivation reported thus far is an approximately twofold increase in the level of myf5 mRNA expression. The presence of normal levels of myogenin and MRF4 transcripts in MyoD–null mice demonstrates that MyoD is not essential for expression of these genes. Mice homozygous for a *myf5*–null mutation also develop normal skeletal muscle, but they die at birth because of the absence of the distal parts of the ribs, which prevents them from breathing (Braun et al. 1992). In contrast, mice lacking both MyoD and myf5 produce no detectable muscle markers and appear to lack skeletal myoblasts (Rudnicki et al. 1993). This phenotype suggests that MyoD and myf5 perform overlapping functions in the genesis of myoblasts.

The phenotype of myogenin–null mice suggests that myogenin acts in a genetic pathway downstream of MyoD and myf5. Mice lacking myogenin possess normal numbers of skeletal myoblasts at the time of birth, but they show a severe reduction of skeletal muscle fibers (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995). The undifferentiated myogenic cells that populate the presumptive muscle-forming regions of myogenin–null mice express MyoD and myf5, indicating that these myogenic factors act at an earlier point than myogenin in the myogenic pathway and suggesting that they cannot direct the formation of normal skeletal muscle in the absence of myogenin. MRF4 is expressed at very low levels in myogenin–null mice (Hasty et al. 1993), consistent with the notion that it acts after myogenin in the myogenic lineage.

To complete the functional analysis of the myogenic bHLH genes, we deleted the *MRF4* gene through homologous recombination in embryonic stem (ES) cells and used these ES cells to create mice lacking MRF4. MRF4–null mice were viable and fertile and showed only a slight reduction in expression of a subset of muscle-specific genes. However, myogenin expression was elevated in adult skeletal muscle from MRF4–null mice, suggesting that it may compensate for the absence of MRF4 and that MRF4 is required for normal down-regulation of myogenin expression. Paradoxically, MRF4–null mice exhibited multiple rib abnormalities, suggesting that expression of MRF4 in skeletal muscle cells affects the outgrowth of rib primordia through an indirect mechanism.

Results

Targeting the MRF4 *locus*

The *MRF4* and *myf5* genes are arranged in a head-to-tail orientation and are separated by ~6 kb in the mouse genome (Braun et al. 1990; Miner and Wold 1990). Like the other vertebrate myogenic bHLH genes, *MRF4* and *myf5* contain three exons, with the bHLH-coding region located in exon 1 (Fig. 1A).

To create a null mutation in the *MRF4* gene, we constructed a replacement targeting vector in which a neomycin resistance gene was inserted in the reverse orientation at codon 69 of *MRF4* and the remainder of the *MRF4* gene was deleted (Fig. 1A). A herpes simplex virus–*thymidine kinase* (HSV–*tk*) gene was placed at the 3′ end of a 5.5-kb genomic DNA fragment encompassing the region between the *MRF4* and *myf5* genes. The targeting vector deleted 1.9 kb of genomic DNA encompassing the segment of the *MRF4* gene that encodes the bHLH region and the entire carboxyl terminus of the protein, but it did not affect the structure of the *myf5* gene. The linearized targeting vector was electroporated into 129 ES cells (McMahon and Bradley 1990), and ES cell clones were isolated after positive–negative selection using G-418 and FIAU.

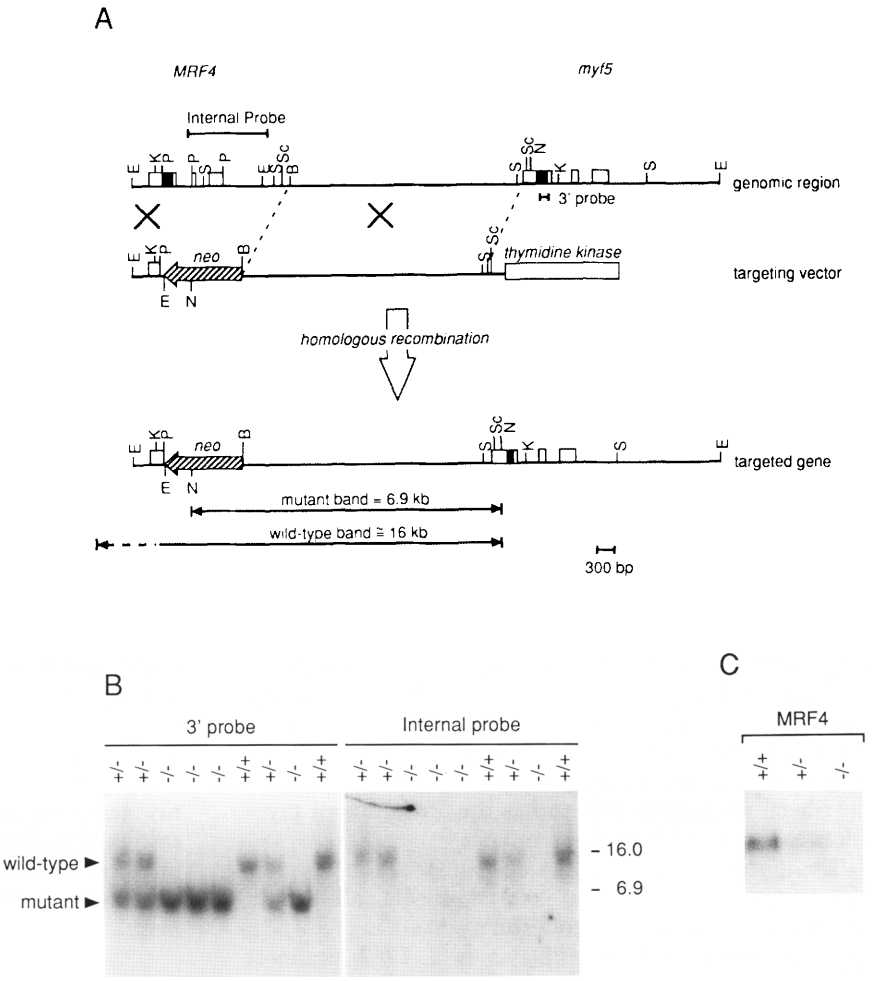

Figure 1. Targeting of the *MRF4* gene. (*A*) Structures of the mouse *MRF4* and *myf5* genes and strategy for inactivation of *MRF4*. The structures of the *MRF4* and *myf5* genes are shown at the *top*. Both genes contain three exons, with the bHLH region (■) encoded by exon 1. Transcription of *MRF4* and *myf5* occurs in the same direction (from *left* to *right* in the diagram). In the targeting vector, (*middle*) the region between the *Pst*I site in exon 1 of *MRF4* and the *Bam*HI site 3′ of the gene was deleted. *pgk–neo* was inserted in the reverse orientation between the *Pst*I and *Bam*HI sites, and HSV-*tk* was cloned at the *Sca*I site at the 5′-untranslated region of *myf5*. Homologous recombination resulted in the deletion of most of *MRF4* but did not affect the structure of *myf5*. The predicted structure of the targeted *MRF4* gene is shown at the *bottom*. Insertion of *neo* introduced *Nco*I and *Eco*RI restriction sites that could be used to distinguish the wild-type and mutant alleles. The positions of the 3′ and internal probes used for Southern blot analyses are indicated. Hybridization of *Nco*I-digested DNA with the 3′ probe yielded fragments of ~16 and 6.9 kb from the wild-type and targeted loci, respectively. Hybridization of the same blots with the internal probe yielded a fragment of 16 kb from the wild-type allele, which was eliminated by the targeted mutation. A 5′ probe outside of the targeted region was also used to confirm targeting events using genomic DNA digested with *Eco*RI (data not shown). (B) *Bam*HI; (E) *Eco*RI; (K) *Kpn*I; (N) *Nco*I; (P) *Pst*I; (S) *Sac*I; (Sc) *Sca*I. (*B*) Southern blots of *Nco*I-digested genomic DNA from a litter of mice arising from an intercross of *MRF4*(+/−) mice. The positions of the 3′ and internal probes are shown in *A*. No hybridization of the internal probe is detected to the mutant gene, from which this region was deleted. (*C*) Analysis of MRF4 mRNA by a Northern blot of RNA from carcasses of newborn mice. Mice heterozygous for the mutant *MRF4* allele have half the amount of MRF4 mRNA as wild-type mice and MRF4–null mice have no detectable MRF4 transcripts. GAPDH mRNA was measured as a constitutive control to ensure equal loading (not shown).

Replacement of the *MRF4* gene with the *neomycin* cassette made it possible to distinguish the targeted *MRF4* allele from the wild-type allele by digestion of genomic DNA with *Nco*I or *Eco*RI and hybridization with labeled probes 5′ and 3′ of the gene, respectively (Fig. 1A). A total of 222 ES cell clones were analyzed for homologous recombination at the *MRF4* locus. Homologous recombination was observed at a frequency of 1:9 in these clones.

Three ES cell clones containing a single targeting event were injected into 3.5-day blastocysts derived from C57Bl/6 mice to produce chimeras. Nine chimeras from two of the ES cell clones transmitted the *MRF4* mutation through the germ line, yielding offspring heterozygous for the *MRF4*–null allele. Intercrosses of these heterozygotes produced offspring homozygous for the *MRF4*–null allele.

Southern blot analysis of genomic DNA from offspring of an intercross of MRF4–null heterozygotes is shown in Figure 1B. Using the 3′ probe, the wild-type and mutant alleles gave rise to bands of ~16 and 6.9 kb, respectively, after digestion with *Nco*I. Hybridization of the same blots with a portion of an *MRF4* cDNA corresponding to the region of deleted genomic DNA confirmed that the predicted portion of the *MRF4* gene had been deleted from the targeted gene (Fig. 1B). Similarly, Northern blot analysis of RNA from carcasses of newborn mice using the same probe showed no MRF4 mRNA in *MRF4*(−/−) offspring (Fig. 1C). There was a twofold reduction in MRF4 mRNA expression in *MRF4*(+/−) offspring, indicating that the wild-type gene was not up-regulated in MRF4 mutants to compensate for the loss of the other allele.

Mice heterozygous for the *MRF4* mutation appeared

normal and gave rise to litters of normal size when they were intercrossed. Genotyping of litters arising from *MRF4*(+/−) intercrosses showed that the *MRF4* mutation was inherited with the predicted Mendelian frequency (Table 1) and that *MRF4*(−/−) mice were fully viable. These mutant mice had body weights comparable to their wild-type littermates, and all of their major muscle groups appeared normal (data not shown).

Expression of muscle structural genes in MRF4–null mice

To determine whether skeletal muscle from MRF4–null mice showed aberrant expression of muscle-specific genes, we measured several muscle-specific transcripts by Northern blot analysis of RNA from skeletal muscle of neonatal and adult mice. Transcripts for muscle creatine kinase (MCK), slow troponin I (TnI), α-skeletal actin, and the δ-subunit of the acetylcholine receptor (AChR) were expressed at comparable levels in muscle from wild-type and MRF4–null neonates (Fig. 2A). In contrast, embryonic myosin heavy chain (MHC) was expressed at a three- to fourfold lower level in muscle from the mutant neonates (Fig. 2A).

In adult skeletal muscle, we observed no significant differences in expression of transcripts for MCK, myosin light chain-1v (MLC-1v), or troponin T (TnT) (Fig. 2B). TnI and α-skeletal actin were also expressed at normal levels in the mutants (data not shown). Similarly, several muscle genes that are expressed preferentially after birth and in adults were expressed at normal levels in MRF4–null adults. These included MHC-2A, MHC-2B, and MHC-2x (data not shown). These results demonstrate that MRF4 is not required for expression of muscle-specific genes at neonatal or adult stages.

MRF4–null mice have a normal distribution of fast- and slow-twitch muscle fibers

To analyze further the potential consequences of the *MRF4*–null mutation on postnatal muscle development, we examined the distribution of fast- and slow-twitch fibers in adult hind limb muscle by immunostaining thin sections with a series of anti-MHC antibodies. Antibodies against adult MHC-2A and MHC-2B, which are expressed in fast-twitch fibers, showed comparable staining patterns in muscle from wild-type and mutant mice (Fig. 3A–D). Similarly, the distribution of slow MHC-expressing muscle fibers was unaffected in MRF4–null mice (Fig. 3E,F). We conclude that MRF4 is not required to establish the normal distribution of adult fast- and slow-twitch fibers.

Table 1. *Genotypes of progeny from MRF4(+/−) intercrosses*

Genotype	Number of mice (%)
+/+	28 (26)
+/−	53 (49)
−/−	27 (25)

The genotypes of offspring from MRF4 (+/−) intercrosses were determined by Southern blot analysis as described in Materials and methods.

Expression of myogenic bHLH genes in MRF4–null mice

We also examined the expression of myogenin, MyoD, and myf5 in MRF4–null mice to determine whether one or more of them might be up-regulated to compensate for the absence of MRF4. Transcripts for these myogenic factors were present at normal levels in skeletal muscle from neonatal mutant mice (Fig. 4A), indicating that MRF4 is not required for their expression before birth. The normal expression of myf5 also suggests that the *MRF4*–null mutation did not result in a *cis* effect on the *myf5* gene.

In adult skeletal muscle, MyoD and myf5 expression were also unaffected by the *MRF4*–null mutation (Fig. 3B). However, myogenin mRNA expression in mutant mice was approximately fourfold that in controls (Fig. 4B). We did not observe an increase in myogenin transcripts in *MRF4*(+/−) mice, suggesting that a twofold decrease in the level of MRF4 is insufficient to alter myogenin expression. The dramatic up-regulation of myogenin expression in MRF4–null mice suggests that MRF4 normally represses myogenin expression after birth and raises the possibility that up-regulation of myogenin compensates for the absence of MRF4 and supports muscle development.

We also examined E9.5 embryos by reverse transcriptase–polymerase chain reaction (RT–PCR) for possible changes in myf5 expression. As shown in Figure 4C, myf5 transcripts were expressed at comparable levels in wild-type and mutant embryos at this stage. Thus, if MRF4 affects myf5 expression, the effect must be subtle.

Somite myogenesis is unaffected in MRF4–null embryos

Because MRF4 is expressed transiently in the somite myotome between E9.0 and E11.5 (Bober et al. 1991; Hinterberger et al. 1991), we analyzed the expression of muscle transcripts in E11.5 embryos by in situ hybridization to determine whether the absence of MRF4 affected early stages of myogenesis in the somite myotome. Myogenin transcripts showed comparable patterns of expression in the somite myotomes, as well as the limb buds, of wild-type and mutant embryos at this stage (Fig. 5A,B). myf5 was also expressed in somites at this stage, and its expression was comparable in wild-type and mutant embryos (Fig. 5C,D). We also measured the expression of myogenin, myf5, and MyoD transcripts in wild-type and mutant day 11.5 p.c. embryos by quantitative RT–PCR and found them to be comparable (data not shown).

To examine the differentiation of myotomal cells, we

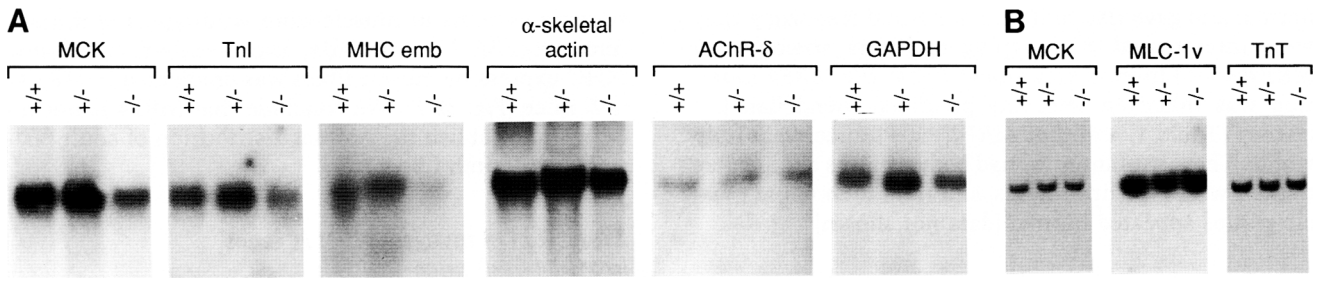

Figure 2. Expression of muscle transcripts in neonatal and adult muscle. RNA was isolated from the carcasses of neonates and from skeletal muscle of adults and was analyzed for expression of the indicated transcripts. Each lane contains RNA from a single animal with the indicated genotype. RNA analyses in *A* were performed by Northern blot and in *B* by quantitative RT–PCR.

looked for the expression of cardiac α-actin transcripts by in situ hybridization; the expression pattern was indistinguishable in wild-type and mutant embryos (Fig. 5E,F). We conclude that deletion of *MRF4* does not result in gross abnormalities in the onset of muscle formation in the somite myotome or limb buds.

MRF4–null mice show defects in rib development

In light of the skeletal defects associated with *myf5*–null and *myogenin*–null mutations (Braun et al. 1992; Hasty et al. 1993; Nabeshima et al. 1993), we stained newborn mice for bone and cartilage with alizarin red and alcian blue, respectively. This revealed the presence of multiple bifurcations and supernumerary processes in the distal regions of the ribs of *MRF4*(−/−) mice (Fig. 6). These anomalies were bilaterally asymmetric; they appeared to occur randomly in different ribs but showed 100% penetrance. MRF4–null mice had a normal number of ribs, and their ossification within the proximal regions nearest the vertebral column appeared normal. However, many of the ribs appeared to emerge from the vertebral bodies at incorrect angles, which may have contributed to some of the fusions that were observed in the distal regions. The correct number of ribs fused with the sternum, and intersternebral cartilage was present where the ribs joined the sternum, indicating that they reached the sternum at approximately the correct time. No other skeletal defects were observed in MRF4–null mice. *MRF4*(+/−) mice did not show rib defects.

To begin to determine when the rib defects occurred during development, we stained day 14 p.c. embryos for

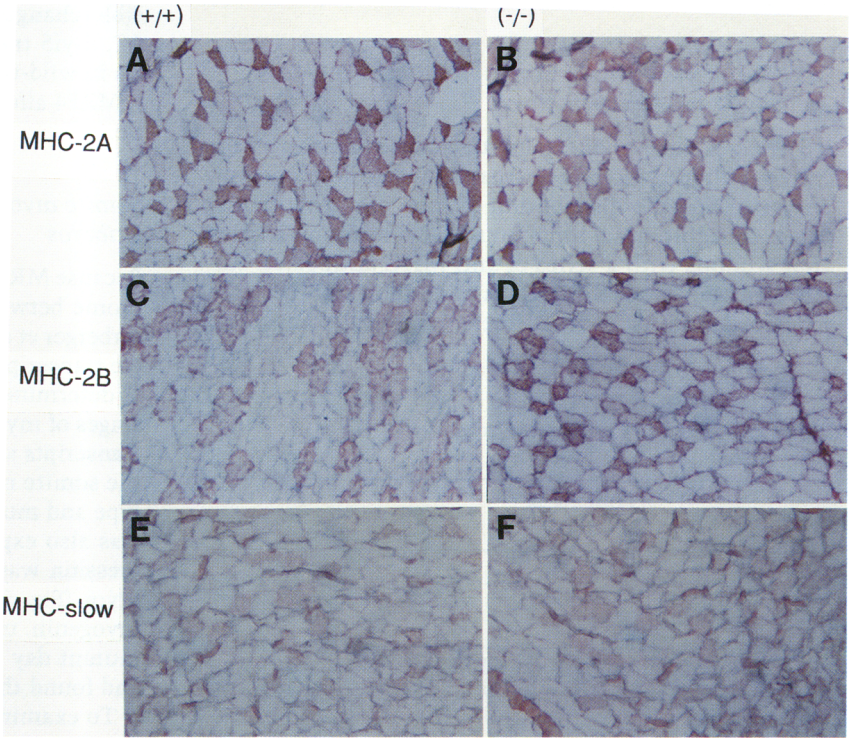

Figure 3. Immunostaining for myosin isoforms in adult muscle from wild-type and MRF4–null mice. Cross sections through the hind limbs of wild-type (*A,C,E*) and MRF4–null (*B,D,F*) mice were stained using antibodies against MHC-2A (*A,B*), MHC-2B (*C,D*), and slow MHC (*E,F*). Magnification, 250×. (*A–D*) Sections through the gastrocnemius; (*E,F*) sections through the soleus.

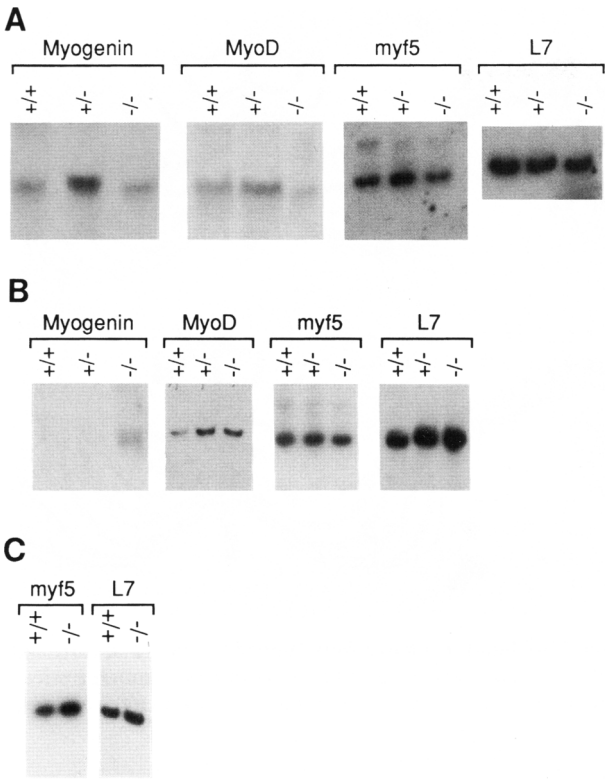

Figure 4. Expression of myogenic bHLH genes in neonatal, adult, and embryonic muscle. RNA was isolated from the carcasses of neonates (*A*), from skeletal muscle of adults (*B*), and from E9.5 embryos (*C*) and was analyzed for expression of the indicated transcripts. Each lane contains RNA from a single animal with the indicated genotype. Expression of myogenin mRNA in *A* and *B* and MyoD mRNA in A was performed by Northern blot analysis. All other transcripts were detected by quantitative RT–PCR. L7 was used to control for equal loading of RNA.

cartilage. At this stage, cartilaginous rib primordia have formed, but ossification has not yet begun. Rib anomalies were apparent in MRF4–null embryos at this stage (not shown), indicating that they arose early during outgrowth of the cartilaginous rib primordia.

Discussion

The expression of MRF4 at high levels in postnatal skeletal muscle has suggested a role for this myogenic bHLH protein in muscle maturation and adult myogenesis (Rhodes and Konieczny 1989; Braun et al. 1990; Miner and Wold 1990). Nevertheless, our results demonstrate that MRF4 is not required for prenatal or postnatal muscle development or for the establishment of the normal distribution of fast- and slow-twitch muscle fibers. The absence of a muscle defect in MRF4–null mice may be explained by the up-regulation of myogenin in adult skeletal muscle of mutant mice and suggests that the functions of myogenin overlap with those of MRF4. Rib defects such as those observed in MRF4–null mice have not been detected in mice with mutations of the other myogenic bHLH genes, indicating that the functions of MRF4 do not entirely overlap those of the other factors.

Myogenin is up-regulated in adult skeletal muscle of MRF4–null mice

We observed no obvious changes in expression of myogenin, MyoD, or myf5 in MRF4 mutant mice before birth. However, myogenin expression was up-regulated markedly in adult skeletal muscle of the mutant mice. What type of mechanism might lead to this increase in myogenin expression in the absence of MRF4? One pos-

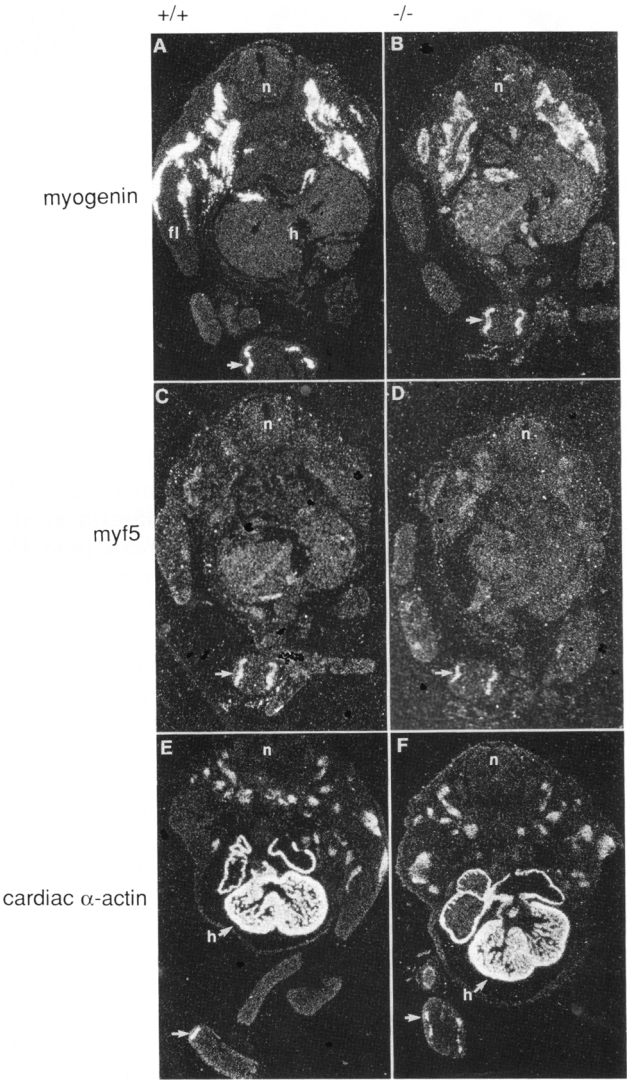

Figure 5. In situ analysis of muscle gene expression in embryos. Transcripts for myogenin (*A,B*), myf5 (*C,D*), and α-cardiac actin (*E,F*) were detected by in situ hybridization of transverse sections of wild-type (*A,C,E*) and mutant (*B,D,F*) embryos at E11.5. The arrow in the lower portion of each panel points to expression in the caudal somite. (fl) Forelimb bud; (h) heart; (n) neural tube.

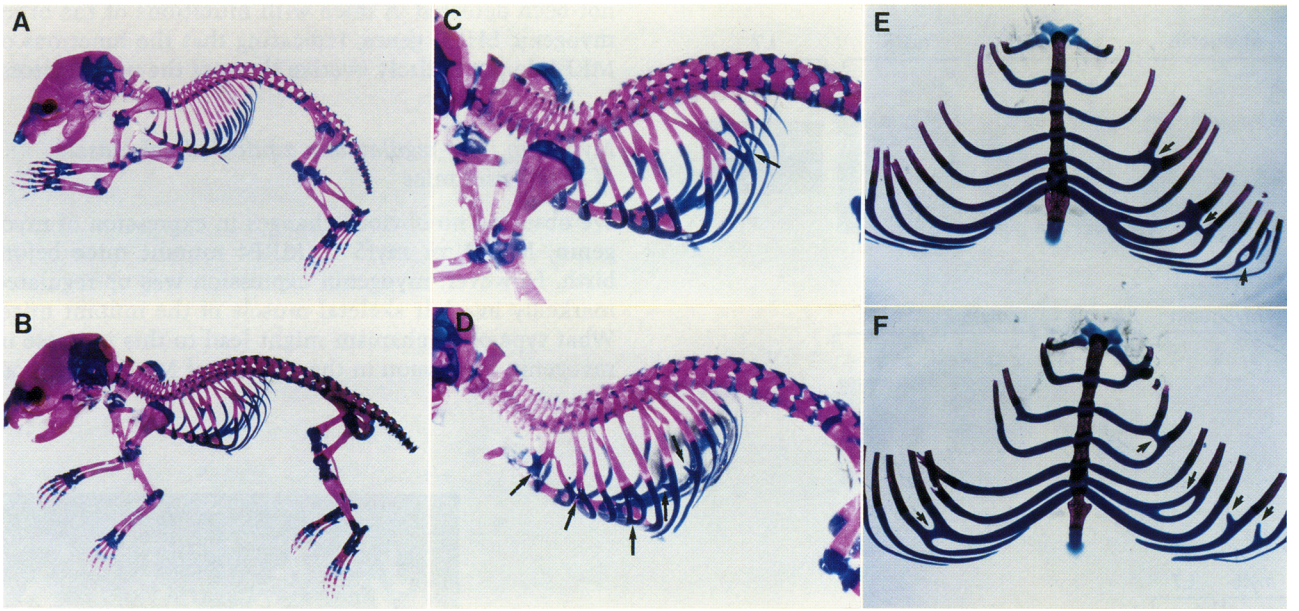

Figure 6. MRF4–null mice show defects in rib development. Wild-type and MRF4–null neonates were stained for bone and cartilage using alizarin red and alcian blue, respectively. (*A*) Wild type; (*B–F*) *MRF4*(−/−) mice; (*C,D*) close-up views of ribs of mutant mice; (*E,F*) frontal view of sternum and ribs of two other mutant mice. Arrows indicate abnormalities, which include bifurcations, fusions, and supernumerary processes.

sibility is that myogenin and MRF4 could mark distinct myogenic lineages, such that in the absence of MRF4, the myogenin-dependent lineage is expanded, resulting in an increase in myogenin-expressing cells. In this regard, MyoD and myf5 have been shown to mark distinct populations of myoblasts in the embryo (Smith et al. 1994; H. Arnold, pers. comm.), and the increase in myf5 expression in MyoD–null mice appears to result from expansion of the myf5-dependent lineage. Alternatively, although probably less likely, MRF4 could normally repress myogenin expression after birth. Such inhibition could be mediated by direct binding of MRF4 to the *myogenin* promoter, which contains an E-box that binds MRF4 with high affinity (Edmondson et al. 1992). In support of this model, we have found that MRF4 is unable to *trans*-activate the *myogenin* promoter (unpubl.).

In addition to its expression in postnatal skeletal muscle, MRF4 is expressed in the somite myotome between E9.0 and E11.5 (Bober et al. 1991; Hinterberger et al. 1991). Myogenin is also expressed in the myotome at this time, and its domain of expression encompasses that of MRF4 (Smith et al. 1994). We did not detect significant differences in expression of myogenin, myf5, MyoD, or α-cardiac actin in the somites of MRF4 mutant mice. Because myogenin expression overlaps with that of MRF4 in the myotome, perhaps it compensates for the absence of MRF4 in the embryo.

Our results, which show that myf5 exhibits normal temporospatial regulation in the absence of MRF4, contrast with the results of a recent study by Braun and Arnold (1995), who found that *myf5* was not expressed in myf6 (MRF4)–null mice. Because of the absence of myf5 expression, their *MRF4* mutation resulted in a phenocopy of the *myf5*–null mutation previously described and was associated with severe rib truncations. The normal expression of myf5 in our mutant mice suggests that the mutation introduced into the *MRF4* gene by Braun and Arnold (1995) disrupted *myf5* expression through a *cis* effect. Their mutation involved the insertion of *phosphoglycerate kinase–neomycin* (*pgk–neo*) in the sense orientation within exon 1 of *MRF4* and the deletion of nucleotides −5 to +207 (codon 40) relative to the transcription initiation site. In our mutation, *pgk–neo* was inserted in the antisense orientation downstream of codon 69, and the remainder of the gene and ~700 bp of 3′-flanking region were deleted. A third deletion mutation has been introduced into the *MRF4* gene by B. Wold and colleagues (B. Wold, pers. comm.). Their mutation results in neonatal lethality and is associated with rib defects that appear to be intermediate between those observed in our mutant mice and those of Braun and Arnold (1995). Together, this range of phenotypes suggests that the *myf5* locus is extremely sensitive to the structure of the *MRF4* gene. Why these mutations have such different consequences on *myf5* expression and rib development remains to be determined.

MRF4 is not required for expression of adult muscle protein isoforms

Recently, it has been suggested that the myogenic bHLH factors may regulate the specific patterns of isoform switching that accompany prenatal and postnatal muscle development (Hughes et al. 1993). Several adult iso-

forms of muscle gene products are regulated in parallel with MRF4 and could be potential targets for regulation by MRF4. The MHC-2A, MHC-2B, and MHC-2x isoforms, for example, are up-regulated after birth (Weydert et al. 1987; DeNardi et al. 1993), but these isoforms were expressed normally in MRF4–null mice. Similarly, the ϵ-subunit of the AChR is up-regulated in parallel with MRF4 after birth (Sunyer and Merlie 1993), but its expression was unaffected in the mutant mice (not shown). There is also a transition from α-cardiac to α-skeletal actin after birth (Minty et al. 1982; Garner et al. 1989), which occurred normally in mutant mice. Thus, it does not appear that MRF4 is required for expression of adult patterns of muscle gene expression. The normal expression of fast MHC-2A and MHC-2B, slow MHC, and slow TnI in MRF4–mutant mice also demonstrates that MRF4 does not regulate fiber type-specific patterns of muscle gene expression.

It is possible that MRF4 regulates certain processes specific to adult skeletal muscle that we have not examined. In this regard, muscle denervation is known to result in up-regulation of AChR subunit genes and the myogenic bHLH genes (Eftemie et al. 1991). We have examined the expression of transcripts for AChR α-subunit and myogenin after denervation of hind limb muscle from MRF4–null mice and have found that they are up-regulated in the same manner as in muscle from normal littermates (W. Zhang, J. Mudd, and E. Olson, unpubl.). Whether MRF4 is required for other processes, such as muscle regeneration, remains to be determined.

MRF4–null mice exhibit rib abnormalities

Unanticipated consequences of the *MRF4*–null mutation were multiple anomalies in the distal portions of the ribs, which included bifurcations, fusions, and supernumerary processes. These defects were observed at all rib levels and were apparently random, with no bilateral symmetry.

How might MRF4 influence rib development? The ribs are derived from cells in the lateral region of the sclerotome that begin migrating at about day 13 p.c. (Rugh 1990). By day 14 p.c., the cartilaginous precursors of the ribs have formed and subsequently become ossified. Rib defects were apparent in MRF4–null mice by day 14 p.c. (not shown), which indicates that they arose as a consequence of an abnormality of cartilage precursors earlier in development. It will be interesting to determine whether the *MRF4*–null mutation alters the expression of the bHLH gene *scleraxis*, which is expressed in the sclerotome and rib primordia (Cserjesi et al. 1995).

MRF4 is normally expressed in the somite myotome between E9.0 and E11.5, which is prior to the time rib progenitors begin migrating from the sclerotome into the body wall (Rugh 1990). Because MRF4 is not expressed in the sclerotome (Bober et al. 1991; Hinterberger et al. 1991), its effects on rib development must be mediated through a non-cell autonomous mechanism, possibly involving the secretion of growth factors or extracellular matrix molecules from muscle cells adjacent to the rib primordia. It is intriguing that MRF4–null mice had bifurcations of the distal ribs, whereas myf5–null mice lack distal ribs. Whether these seemingly opposite effects on rib development reflect the involvement of these genes in a common mechanism of rib development remains to be determined.

Possibilities for genetic redundancy

The inactivation of *MRF4* now completes the analysis of the individual roles of the four myogenic bHLH genes in muscle development. The phenotypes of the different mutant mice are consistent with the model shown in Figure 7. According to this model, MyoD and myf5, which are expressed in proliferating myoblasts, are involved in the generation of myoblasts from mesodermal precursors in the somites, whereas myogenin and MRF4, which are expressed specifically in differentiated muscle cells, are involved in activation of muscle structural genes. *MRF4* is thought to lie downstream of myogenin

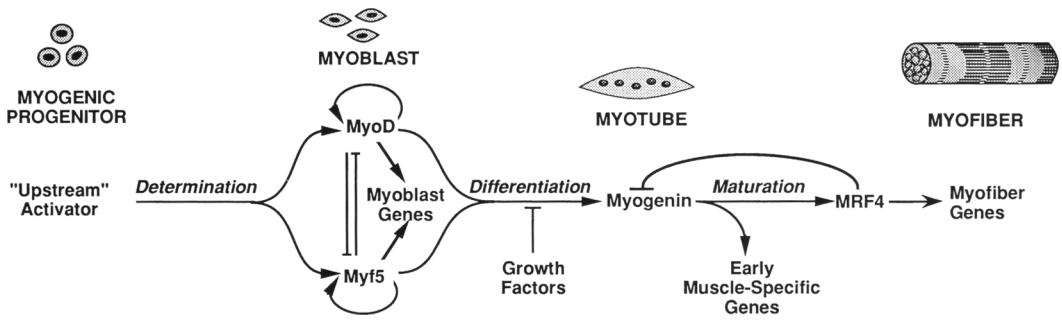

Figure 7. Schematic of the pathway of myogenic regulatory genes. MyoD and myf5 play redundant roles in establishing myoblast identity and are responsible for activation of myogenin expression. Myogenin is expressed early in the muscle differentiation pathway and is required for normal muscle development in vivo. MRF4 is expressed after myogenin and is the predominant myogenic regulatory gene expressed in adult skeletal muscle. Myogenin is normally down-regulated when MRF4 is up-regulated after birth. In MRF4–null mice, myogenin continues to be expressed at a high level, suggesting that MRF4 is normally required for its down-regulation. Adapted from Olson and Klein (1994).

in this myogenic pathway because it is normally expressed after myogenin (Rhodes and Konieczny 1989; Braun et al. 1990; Miner and Wold 1990) and because it is expressed at low levels in myogenin–null mice (Hasty et al. 1993). Further evidence that *MRF4* lies downstream of myogenin comes from the observation that the *MRF4* gene promoter is *trans*-activated efficiently by myogenin (Black et al. 1995). Our results indicate that in the absence of MRF4, myogenin continues to be expressed at high levels and can support normal muscle development.

Although the phenotype of MRF4–null mice demonstrates that MRF4 is not required for muscle development and suggests that its functions overlap with those of myogenin, we consider it unlikely that the functions of MRF4 entirely overlap those of myogenin or other myogenic bHLH factors; if they did, the *MRF4* gene would have sustained mutations during evolution attributable to the lack of selection pressure. However, the *MRF4* genes from mice (Miner and Wold 1990), rat (Rhodes and Konieczny 1989), human (Braun et al. 1990), chicken (Fujisawa-Sehara et al. 1992), and frog (Jennings 1992) are highly homologous and their expression patterns late in the myogenic program are similar. Now that all four myogenic bHLH genes have been inactivated in transgenic mice, it will be possible to assess further the extent to which their functions overlap by combining the different mutations.

Materials and methods

Creation of the MRF4 *targeting vector*

A genomic clone encompassing the *MRF4* and *myf5* genes was isolated from a BALB/c mouse genomic library. The targeting vector was constructed by cloning into Bluescript (Stratagene) a genomic restriction fragment extending from an *Eco*RI site at bp −292 relative to the *MRF4* transcription start site to a *Pst*I site at codon 69 in the *MRF4* gene. A *neo* resistance cassette under control of the *pgk* promoter was then inserted in the reverse orientation into the same *Pst*I site. A genomic restriction fragment extending from a *Bam*HI site 625 bp 3′ of an *Eco*RI site near the end of the *MRF4* gene to a *Sca*I site in the 5′-untranslated region of the *myf5* gene was then ligated to the 5′ end of the *neo* resistance cassette. Finally, a *tk* gene under control of the herpes simplex virus promoter, pMC1–HSVtk (Mansour et al. 1988), was cloned into an *Xho*I site in the polylinker of Bluescript immediately 3′ of the long arm of genomic homology. All of the cloning junctions in the targeting vector were confirmed by DNA sequencing. The *MRF4* targeting vector was linearized by digestion with *Not*I before electroporation.

Generation of MRF4*–null mice*

The *MRF4* targeting vector was electroporated into 129 ES cells (McMahon and Bradley 1990) using a Bio-Rad gene pulser (500 μF, 240 V), and cells were plated on feeder layers of SNL76/7 cells in the presence of G-418 (800 μg/ml) and FIAU (200 μM) for positive-negative selection. There was a fourfold enrichment for targeting events in the presence of FIAU. After selection, surviving clones were isolated and replica plated onto SNL76/7 fibroblasts in 96-well microtiter plates. Southern blot analysis was performed on the individual clones as described (Ramirez-Solis et al. 1992), using DNA digested with *Nco*I and a probe 3′ of the gene or one within the deleted region. The 3′ probe was a 240-bp *Sca*I–*Nco*I fragment from the first exon of *myf5* (see Fig. 1A). The internal probe was a *Pst*I–*Sac*I fragment from within the gene. A 625-bp *Eco*RI–*Pst*I fragment from the first exon of *MRF4* was used as a 5′ probe and was hybridized to DNA digested with *Eco*RI (data not shown).

Three independent ES cell clones containing a targeted *MRF4* allele were expanded and injected into 3.5-day mouse embryos, which were in turn reimplanted into foster mothers to generate high percentage of chimeras. Chimeras obtained from two of the ES cell clones transmitted the mutant *MRF4* allele through the germ line.

Genotyping of progeny

Mice carrying the *MRF4* mutation were identified by Southern blot analysis of genomic DNA isolated from tail biopsies of neonates and from yolk sacs of embryos. Genomic DNA was isolated by incubation of tissue in lysis buffer [10 mM Tris (pH 8.0), 25 mM EDTA (pH 8.0), 100 mM NaCl, 1% SDS, 0.2 mg/ml of proteinase K] at 50°C for 3 hr, followed by chloroform–phenol extraction and ethanol precipitation. After restriction enzyme digestion, genomic DNA was fractionated on a 0.7% agarose gel, transferred to Zeta-probe GT membranes, and hybridized with ^{32}P-labeled probes. Genomic DNA was digested with *Nco*I and hybridized to 3′ and internal probes as described above.

RNA isolation and analysis

Total RNA was isolated from the carcasses of neonates after removal of the heads and internal organs and from the skeletal muscle of adults, using the guanidinium isothiocyanate procedure as described (Edmondson and Olson 1989). After separation on a 1% agarose gel and transfer to Zeta-probe GT membranes, blots were hybridized to ^{32}P-labeled probes. Sources of probes were mouse myogenin (Edmondson and Olson 1989), mouse MyoD (Davis et al. 1987), rat MRF4 (Rhodes and Konieczny 1989), mouse MCK (Buskin et al. 1985), rat embryonic MHC (Medford et al. 1980), AChR-δ-subunit (LaPolla et al. 1984), slow troponin I (Koppe et al. 1989), α-skeletal actin (Hu et al. 1986), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort et al. 1985).

RT–PCR was performed using total RNA from muscle as described previously (Martin et al. 1994). Twenty cycles of PCR were used for all reactions; it was determined that the PCR was in the linear range for ≤23 cycles of amplification. The following primers were used: myf5 5′-primer, TGTATCCCCTCACCAGAGGAT, and 3′-primer, GGCTGTAATAGTTCTCCACCTGTT (Hannon et al. 1992); MCK 5′-primer, CAATAAGCTTCGCGATAAGGAG, and 3′-primer, GATGGGATCAAACAGGTCCTTG (Hollenberg et al. 1993); MLC-1v 5′-primer, TCAGGAAGCCCAGGGCAGGC, and 3′-primer, CTTCCTCACCAAGCCTGAGG (Miller-Hance et al. 1993); and TnT 5′-primer, CTTACTGCTCCTAAGATCCCG, and 3′-primer, CTGGCGCTCCCGCTCCTTCTC (Breitbart and Nadal-Ginard 1987). The MyoD, myogenin, and L7 primers have been described previously (Hollenberg et al. 1993).

In situ hybridization

Mouse embryos were fixed and embedded for in situ hybridization as described (Lyons et al. 1990). Briefly, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline. After dehydration and infiltration with paraffin, 5- to 7-μm sections were cut on a microtome and mounted on gelatinized glass

slides. Sections were then deparaffinized in xylene, rehydrated, treated with proteinase K, postfixed, treated with triethanolamine/acetic anhydride, washed, and dehydrated.

Sources of probes for in situ hybridization were myogenin (Edmondson and Olson 1989) and α-cardiac actin (Sassoon et al. 1988). For myf5, we cloned a *SacI–KpnI* fragment from the first exon of the mouse *myf5* gene into the Bluescript II SK vector, which was then digested with *Pvu*II before in vitro transcription with T7 polymerase.

cRNA transcripts were labeled with [^{35}S]UTP using an in vitro transcription kit (Stratagene) according to the manufacturer's instructions (Venuti et al. 1995).

Skeletal analysis

Neonatal mice and embryos were eviscerated and placed in water overnight. Carcasses were then immersed in a 65°C water bath for 1 min, fixed in ethanol for 3 days, and stained with alcian blue [15 mg alcian Blue 8Gx (Sigma), 80 ml of 95% ethanol, and 20 ml of glacial acetic acid] for 8–12 hr. Skeletons were then rinsed in ethanol overnight and cleared in 2% KOH for 6 hr. Counterstaining for bone was performed using alizarin red (Sigma, 50 mg/liter in 2% KOH) for 3 hr. Skeletons were then cleared in 2% KOH and stored in 100% glycerol.

Histology

Sectioning of frozen tissue and immunostaining with anti-MHC antibodies was performed as described (Schiaffino et al. 1986). Sections 10 μm thick were stained with the following antibodies: anti-MHC-2A (antibody SC71; Schiaffino et al. 1989), anti-MHC-2B (antibody BF-F3; Schiaffino et al. 1989), and anti-slow MHC (antibody BAF8; Maier et al. 1988). Antibody was visualized using the Histostain-SP kit (Zymed Laboratories, Inc.), which involves biotinylated secondary antibody and horseradish peroxidase–streptavidin conjugate.

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