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/hecuculin/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).
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; Tajbaksh 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.


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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 (I) 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 PstI site in exon 1 of MRF4 and the BamHI site 3' of the gene was deleted. pgk-neo was inserted in the reverse orientation between the PstI and BamHI sites, and HSV-tk was cloned at the ScaI 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 NcoI and EcoRI 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 NcoI-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 3' probe outside of the targeted region was also used to confirm targeting events using genomic DNA digested with EcoRI [data not shown]. (B) BamHI, [E] EcoRI, [K] KpnI, [N] Ncol, [P] PstI, [S] ScaI, [Sc] SacI. (B) Southern blots of NcoI-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 NcoI or EcoRI and hybridization with labeled probes 3' 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 NcoI. 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 were expressed at comparable levels in muscle from wild-type and 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.

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 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

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.
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 \( \text{MRF4} \) 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 \( \text{myf5-null} \) and \( \text{myogenin-null} \) 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 \( \text{MRF4}^{-/-} \) 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 intersternobranchial 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...
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 post-

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
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 phenotype 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-

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.
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-null 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 EcoRI site at bp –292 relative to the MRF4 transcription start site to a PstI 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 PstI site. A genomic restriction fragment extending from a BamHI site 625 bp 3' of an EcoRI site near the end of the MRF4 gene to a SacI 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 XhoI 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 NotI before electroporation.

Generation of MRF4–null mice

The MRF4 targeting vector was electroporated into 129 ES cells (McManus 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 NcoI and a probe 3' of the gene or one within the deleted region. The 3' probe was a 240-bp Scal–NcoI fragment from the first exon of myf5 [see Fig. 1A]. The internal probe was a PstI–SacI fragment from within the gene. A 625-bp EcoRI–PstI fragment from the first exon of MRF4 was used as a 5' probe and was hybridized to DNA digested with EcoRI (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 reimplemented 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 to 32p-labeled probes. Sources of probes were mouse myogenin (Edmondson and Olson 1989), MRF4 (Rhodes and Konieczny 1989), mouse MCK (Buskin et al. 1985), rat embryo MHC (Medford et al. 1980), AChR–β-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, TGTATCCCCTACCATGGA, and 3'-primer, GGCTGTAATAGTTCTGCACCCCTGTT (Hannon et al. 1992); MCK 5'-primer, CTTACTGCTCCTAAGATCCCG, and 3'-primer, GATGGCTCCTAGCCATCGATG, and TnT 5'-primer, CTTCCTACAAAGCTCTAGG (Miller-Hance et al. 1993); and TnT 5'-primer, CTACCCTCAGCGTCTTCTCG (Bretthart 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 paraaffin, 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 protease 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 PvuII before in vitro transcription with T7 polymerase.

cRNA transcripts were labeled with [35S]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 hr, fixed in ethanol for 3 days, and stained with alcin blue [15 mg alcin 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), and anti-slow MHC (antibody BAFl; 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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References

Black, R., J. Martin, and E.N. Olson. 1995. The mouse MRF4 promoter is transactivated directly and indirectly by muscle-specific transcription factors. J. Biol. Chem. 270: 2889–2892.

Bober, E., G.E. Lyons, T. Braun, G. Cossu, M. Buckingham, and H.H. Arnold. 1991. The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. J. Cell Biol. 113: 1255–1265.

Braun, T. and H.H. Arnold. 1995. Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. EMBO J. 14: 1176–1186.

Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H.H. Arnold. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. 8: 701–709.

Braun, T., E. Bober, B. Winter, N. Rosenthal, and H.H. Arnold. 1990. MyoD-6, a new member of the human gene family of myogenic determination factors: Evidence for a gene cluster on chromosome 12. EMBO J. 9: 821–831.

Braun, T., M.A. Rudnicki, H.H. Arnold, and R. Jaenisch. 1992. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell 71: 369–382.

Braun, T., E. Bober, M.A. Rudnicki, R. Jaenisch, and H.H. Arnold. 1994. MyoD expression marks the onset of skeletal myogenesis in Myf-5 mutant mice. Development 120: 3083–3092.

Breitbart, R.E. and B. Nadal-Ginard. 1987. Developmentally induced, muscle-specific trans factors control the differential splicing of alternative and constitutive troponin T exons. Cell 49: 793–803.

Buckingham, M. 1992. Making muscle in mammals. Trends Genet. 8: 144–148.

Buskin, J.N., J.B. Jaynes, J.S. Chamberlain, and S.D. Hauschka. 1985. The mouse muscle creatine kinase cDNA and deduced amino acid sequences: Comparison to evolutionarily related enzymes. J. Mol. Evol. 22: 334–341.

Chakraborty, T. and E.N. Olson. 1991. Domains outside of the DNA-binding domain impart target gene specificity to myogenin and MRF4. Mol. Cell. Biol. 11: 6103–6108.

Chakraborty, T., T.J. Brennan, and E.N. Olson. 1991. Differential trans-activation of a muscle-specific enhancer by myogenic helix-loop-helix proteins is separable from DNA binding. J. Biol. Chem. 266: 2878–2882.

Cheng, T.C., M. Wallace, J.P. Merlie, and E.N. Olson. 1993. Separable regulatory elements govern myogenin transcription in embryonic somites and limb buds. Science 261: 215–218.

Chevallier, A., M. Kieny, and A. Mauger. 1977. Limb-somite relationship. Origin of the limb musculature. J. Embryol. Exp. Morphol. 41: 245–258.

Christ, B., H. Jacob, and M. Jacob. 1977. Experimental analysis of the origin of the wing musculature in avian embryos. Anat. Embryol. 150: 171–186.

Cserjesi, P., D. Brown, K.L. Ligon, G. Lyons, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, and E.N. Olson. 1995. Scleraxis: A basic-helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. Development 121: 1099–1110.

Davis, R.L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51: 987–1000.

DeNardi, C., S. Ausoni, P. Moretti, L. Gorza, M. Velleca, M. Buckingham, and S. Schiaffino. 1993. Type 2X myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene. J. Cell Biol. 123: 823–835.

Edmondson, D.G. and E.N. Olson. 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes & Dev. 3: 628–640.

Edmondson, D.G., T.-C. Cheng, P. Cserjesi, T. Chakraborty, and E.N. Olson. 1992. Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. Mol. Genes & Development 1397
Eftemie, R., H.R. Brenner, and A. Buonanno. 1991. Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity. *Proc. Natl. Acad. Sci.* 88: 1349–1353.

Emerson, C.P., Jr. 1990. Myogenesis and developmental control genes. *Curr. Opin. Genet. Dev.* 2: 2165–2175.

Fort, P., L. Marty, M. Piekaczyczak, S. Sabrouy, C. Dani, P. Jeantuer, and J.M. Blanchard. 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res.* 13: 1431–1442.

Fujisawa-Sehara, A., Y. Nabeshima, T. Komiya, T. Uetsuki, A. Asakura, and Y.-I. Nabeshima. 1992. Differential trans-activation of muscle-specific regulatory elements including the myosin light chain box by chicken MyoD, myogenin, and MRF4. *J. Biol. Chem.* 267: 10031–10038.

Garner, I., D. Sassoon, J. Vanderkerkhove, S. Alonso, and M. Buckingham. 1989. A developmental study of the abnormal expression of α-cardiac and α-skeletal actins in the striated muscle of a mutant mouse. *Dev. Biol.* 134: 236–245.

Hannon, K., C.K. Smith, K.R. Bales, and R.F. Santerre. 1992. Temporal and quantitative analysis of myogenic regulatory and growth factor gene expression in the developing mouse embryo. *Dev. Biol.* 151: 137–144.

Hasty, P., A. Bradley, J.H. Morris, J.M. Venuti, and E.N. Olson. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* 364: 501–506.

Hintenberger, T.J., D.A. Sassoon, S.J. Rhodes, and S.F. Konieczny. 1991. Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev. Biol.* 147: 144–156.

Hollenberg, S.M., P.F. Cheng, and H. Weintraub. 1993. Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. *Proc. Natl. Acad. Sci.* 90: 8028–8032.

Hu, M.C.T., S.B. Sharp, and N. Davidson. 1986. The complete sequence of the mouse skeletal α-actin gene reveals several conserved and inverted repeat sequences outside of the protein coding region. *Mol. Cell. Biol.* 6: 15–25.

Hurst, P., S.M. Taylor, S.J. Tapscott, C.M. Gurley, W.J. Carter, and C.A. Peterson. 1993. Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development* 118: 1137–1147.

Jan, Y.N. and L.Y. Jan. 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. *Cell* 75: 827–830.

Jennings, C.G.B. 1992. Expression of the myogenic gene MRF4 during *Xenopus* development. *Dev. Biol.* 150: 121–132.

Koppe, R.L., P.L. Hallauer, G. Karpati, and K.E.M. Hasting. 1989. cDNA clone and expression analysis of rodent fast and slow troponin I mRNAs. *J. Biol.Chem.* 264: 14327–14333.

LaPolla, R.D., K.M. Mayne, and N. Davidson. 1984. Isolation and characterization of a cDNA clone for the complete protein coding region of the delta subunit of the mouse acetylcholine receptor. *Proc. Natl. Acad. Sci.* 81: 7970–7974.

Lyons, G.E., S. Schiaffino, D.A. Sassoon, M. Barton, and M. Buckingham. 1990. Developmental regulation of myogenin gene expression in mouse cardiac muscle. *J. Cell Biol.* 111: 2427–2436.

Maier, A., L. Gorza, S. Schiaffino, and D. Pette. 1988. A combined histochemical and immunohistochemical study on the dynamics of fast-to-slow fiber transformation in chronically stimulated rabbit muscle. *Cell Tissue Res.* 254: 59–68.

Mak, K.-L., R.Q. To, Y. Kong, and S.F. Konieczny. 1992. The MRF4 activation domain is required to induce muscle-spe-
Embryonic myosin heavy chain as a differentiation marker of developing human skeletal muscle and rhabdomyosarcoma. A monoclonal antibody study. *Exp. Cell Res.* **163**:211–220.

Schiaffino, S., L. Gorza, S. Sartore, L. Saggini, S. Ausoni, M. Vianello, K. Gundersen, and T. Lomo. 1989. Three myosin heavy chain isoforms in type 2 skeletal muscle fibers. *J. Muscle Res. Cell Motil.* **10**:197–205.

Smith, T.H., A.M. Kachinsky, and J.B. Miller. 1994. Somite subdomains, muscle cell origins, and the four muscle regulatory factor proteins. *J. Cell Biol.* **127**:95–105.

Sunyer, T. and J.P. Merlie. 1993. Cell type- and differentiation-dependent expression from the mouse acetylcholine receptor ε-subunit promoter. *J. Neurosci. Res.* **36**:224–234.

Tajbakhsh, S. and M. Buckingham. 1994. Mouse limb muscle is determined in the absence of the earliest myogenic factor myf-5. *Proc. Natl. Acad. Sci.* **91**:747–751.

Venuti, J.M., J.S. Morris, J.L. Vivian, E.N. Olson, and W.H. Klein. 1995. Myogenin is required for late but not early aspects of myogenesis during mouse development. *J. Cell Biol.* **128**:563–576.

Wachtler, F. and B. Christ. 1992. The basic embryology of skeletal muscle formation in vertebrates: The avian model. *Semin. Dev. Biol.* **3**:217–227.

Weintraub, H. 1993. The MyoD family and myogenesis: Redundancy, networks, and thresholds. *Cell* **75**:1241–1244.

Weintraub, H., R.L. Davis, S.J. Tapscott, M. Thayer, M. Krause, R. Benezra, K.T. Blackwell, D. Turner, R. Rupp, S.M. Holenberg, Y. Zhuang, and A.B. Lassar. 1991. The myoD gene family: Nodal point during specification of the muscle cell lineage. *Science* **251**:761–766.

Weydert, A., P. Barton, A.J. Harris, C. Pinset, and M. Buckingham. 1987. Developmental pattern of mouse skeletal myosin heavy chain gene transcripts in vivo and in vitro. *Cell* **49**:121–129.

Wright, W.E., D.A. Sassoon, and V.K. Lin. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56**:607–617.

Yutzey, K.E., S.J. Rhodes, and S.F. Konieczny. 1990. Differential trans-activation associated with the muscle regulatory factors MyoD1, myogenin and MRF4. *Mol. Cell. Biol.* **10**:3934–3944.
Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies.

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References
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