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Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development

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Myf-6, alternatively called MRF4 or herculin, is a member of a group of muscle-specific transcription factors which also comprises Myf-5, myogenin and MyoD. All family members show distinct expression patterns during skeletal muscle development and can convert a variety of cell lines to myocytes. We disrupted the Myf-6 gene in mice to investigate its functional role in the network of regulatory factors controlling myogenesis. Homozygous mice carrying the disrupted Myf-6 gene show pronounced down-regulation of Myf-5 transcription for reasons presently unknown. Consequently, these mice represent a double knock-out model for Myf-6 and Myf-5. The mutants resemble most of the Myf-5 phenotype with aberrant and delayed early myotome formation and lack of distal rib structures. In addition, we find a reduction in the size of axial muscles in the back. Apart from changes in the pattern of some contractile protein isoforms, the existing myofibers appear fairly normal. This suggests that Myf-6 has no major role in the maturation of myotubes, as previously proposed. Our results provide evidence that skeletal myogenesis can proceed in the absence of two myogenic factors, Myf-5 and Myf-6, therefore they must exert largely non-redundant functions *in vivo*.

Key words: development/mouse/Myf

Introduction

A family of muscle-specific transcription factors which belongs to the superfamily of basic helix–loop–helix (bHLH) proteins has been shown to play an important role in regulating myogenesis *in vitro* and *in vivo* (reviewed by Weintraub *et al.*, 1991; Arnold and Braun, 1993a; Emerson, 1993; Olson and Klein, 1994). Members of this group include MyoD (Davis *et al.*, 1987), myogenin, (Braun *et al.*, 1989b; Edmondson and Olson, 1989; Wright *et al.*, 1989), Myf-5 (Braun *et al.*, 1989a) and Myf-6 (Braun *et al.*, 1990a), independently isolated as MRF4 (Rhodes and Konieczny, 1989) and herculin (Miner and Wold, 1990). Each of these proteins has the ability to induce the myogenic phenotype when constitutively expressed from transfected expression vectors in permissive cell lines (Weintraub *et al.*, 1991). Very little is known, however, of how this process works in detail and which primary genes are switched on during determination and differentiation (reviewed by Weintraub, 1993). Although numerous muscle-specific genes have been identified which contain one or more copies of the canonical E-box motif, the general DNA binding site for bHLH proteins, it is not clear whether all or most of these sites represent natural targets for myogenic factors and in what context these genes are directly activated by myogenic bHLH factors (Davis *et al.*, 1990; Braun and Arnold, 1991). MyoD, myogenin and Myf-5 when expressed at high concentrations in transient transfection experiments activitate E-box-containing reporter genes and endogenous muscle gene promotors. Myf-6 may have a different transactivating capacity, since it activates only some, but not all, E-box-containing, muscle-specific reporter constructs (Yutzey *et al.*, 1990; Chakraborty *et al.*, 1991).

In transfected cells, myogenic bHLH proteins are capable of inducing their own expression by auto- and cross-activation (Braun *et al.*, 1989b; Thayer *et al.*, 1989). The importance of this *in vitro* phenomenon for natural muscle development is unclear, but it is reasonable to assume that it may play a role in amplifying gene expression, thereby maintaining the stable muscle phenotype (Weintraub, 1993). For the myogenin gene it has been demonstrated that it constitutes a primary target for activation by MyoD and Myf-5 in cell culture (Hollenberg *et al.*, 1993; Buchberger *et al.*, 1994). Lack of early myogenin expression in Myf-5-deficient mice confirms that cross-activation is also an important regulatory principle *in vivo* (Braun *et al.*, 1994). In fact, myogenin promotor elements have been analyzed and found to contain E-box and RSRF binding sites (Cheng *et al.*, 1992, 1993; Yee and Rigby, 1993). In contrast, detailed information is not available on the control regions of the Myf-6 and Myf-5 genes. The available transgenic mouse strains carrying promotor constructs for Myf-5 and Myf-6 reproduce only some aspects of the natural expression pattern (Patapoutian *et al.*, 1993). Most likely this is due to a complicated regulation of these genes, with important control elements located in distant positions outside the genes.

All skeletal muscles in vertebrates are derived from somites and the prechordal plate mesoderm (Christ *et al.*, 1978; Buckingham, 1992). In the mouse, the first primary skeletal muscle myocytes can be detected at E8.5 in cranial somites (Fürst *et al.*, 1989). Around E14 these primary myocytes begin to be replaced by secondary myotubes which utilize the primary muscle cells as a scaffold to form mature muscle (Duxson *et al.*, 1989). The expression of myogenic factors during mouse myogenesis has mainly been assessed by *in situ* hybridization experiments, which identified Myf-5 as the first myogenic factor expressed in E8.0 somites (Ott *et al.*, 1991), immediately followed by myogenin (E8.5) and Myf-6

(E9.0) (Bober *et al.*, 1991; Hinterberger *et al.*, 1991). MyoD appears last, at E10.5 (Sassoon *et al.*, 1989). Both Myf-5 and Myf-6 show a transient expression pattern and transcripts cannot be detected by *in situ* hybridization after E14, although they are still readily detectable by Northern blot analysis and RNase protection (Bober *et al.*, 1991; Braun *et al.*, 1992b; Rudnicki *et al.*, 1992). At E16 the level of Myf-6 mRNA increases again in all muscles, until it becomes the prevalent myogenic factor at birth (Bober *et al.*, 1991; Hinterberger *et al.*, 1991).

Expression of MyoD and Myf-5 in cultured myoblast cell lines is mainly confined to undifferentiated cells (Braun *et al.*, 1989b; Thayer *et al.*, 1989). In contrast, myogenin is found only in differentiated myocytes and Myf-6 appears after prolonged differentiation periods *in vitro* (Wright *et al.*, 1989; Miner and Wold, 1990). The different expression patterns of myogenic factors *in vitro* and *in vivo* have raised speculation about possible functional differences between these factors. Indeed, recent inactivations of myogenic bHLH genes in mice provided evidence for such functional differences among individual myogenic factors. As we have shown previously, mutation of the Myf-5 gene leads to a delay in myotome differentiation until MyoD expression commences normally (Braun *et al.*, 1992b, 1994). Following initiation of MyoD expression in Myf-5 mutant mice, skeletal muscle development proceeds seemingly normally and muscle tissue in mutants cannot be distinguished from that of normal mice at birth. In addition, Myf-5 mutant animals suffer from a severe rib defect leading to perinatal death (Braun *et al.*, 1992b). In contrast, MyoD-deficient mice are viable and fertile and display no morphological abnormalities. They do exhibit, however, a moderate increase in the amount of Myf-5 RNA (Rudnicki *et al.*, 1992). Inactivation of both the MyoD and Myf-5 genes results in mice which are completely devoid of myofibers and, presumably, myoblasts (Rudnicki *et al.*, 1993). In contrast, targeted mutation of the myogenin gene leads to a partial block of myoblast differentiation (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). The resulting muscles contain only a few myofibers, but apparently normal numbers of mononucleated myoblasts. Interestingly, the mutant myoblasts express MyoD and readily differentiate into myotubes in culture (Nabeshima *et al.*, 1993).

The available data from knock-out mice models allows placement of the different myogenic factors into a genetic pathway, with Myf-5 and/or MyoD controlling determination of myoblasts and myogenin regulating terminal differentiation (Arnold and Braun, 1993b; Olson and Klein, 1994). The role of Myf-6, the fourth myogenic factor in this network, remains unknown. Therefore, we inactivated the Myf-6 gene in mice in order to study its interaction with the other myogenic factors and its possible impact on the muscle differentiation process. We observed an unexpected loss of Myf-5 expression, which presumably causes abnormal somite development and the rib phenotype. In addition, we noticed a considerable reduction in back muscle formation. Moreover, expression of embryonic myosin heavy chain (emb MyHC) was drastically decreased, along with alterations in other contractile protein isoforms.

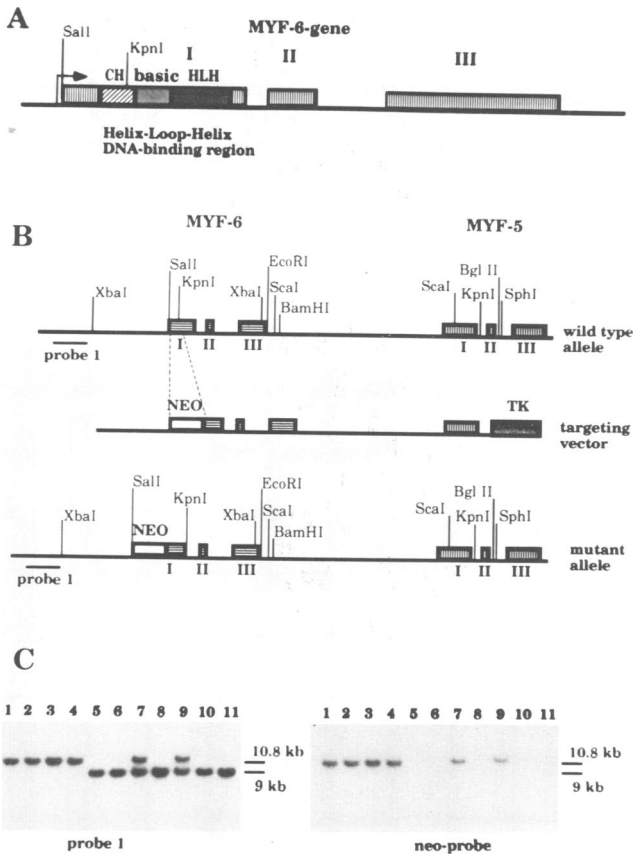

Fig. 1. Targeted disruption of the Myf-6 gene. (**A**) Schematic representation of the Myf-6 gene. The gene consists of three exons. The first exon encodes the basic helix−loop−helix DNA binding domain, a cysteine/hystidine-rich region and an activation domain. (**B**) Schematic diagram of the targeting procedure used to disrupt the Myf-6 gene. The top line shows the genomic structure of the Myf-6/Myf-5 locus. The targeting vector is shown in the middle. The *neo* expression cassette, including the phosphoglycerate kinase promotor and a polyadenylation signal, was inserted into exon 1 of the Myf-6 gene, removing nucleotides −5 to +207. The bottom line represents the structure of the disrupted gene. A *pgk*/HSV thymidine kinase expression cassette (TK) was included in the vector to allow double selection. (**C**) The wild-type (9 kb) and mutant alleles (10.8 kb) are shown on a Southern blot performed with DNA from a cross of heterozygous Myf-6 parents using probe 1 and a *neo* probe for hybridization. Note that the mutant, but not the wild-type, allele can be detected with the *neo* probe.

Results

Inactivation of the Myf-6 gene in ES cells and generation of mouse strains

Three different constructs were utilized to target the Myf-6 gene. The first two vectors HRCmyf6#10A and HRCmyf6#14B, which were derived from a 129/t^{AE5} genomic library, gave very few or no homologous recombinants, although DNA from the same library was used to target the Myf-5 gene at high frequency. The low recombination frequency of the initial two vectors prompted us to re-isolate the Myf-6 gene from a cosmid library made from J1-ES cells and to construct a third plasmid HRCmyf6#19C. The design of the vector was identical to HRCmyf6#14B. This final targeting vector HRCmyf6#19C contained the Myf-6 gene in which nucleotides −5 to +207 relative to the transcription start site were replaced by the *pgk−neo* cassette in the sense

Table I. Homologous recombination frequencies in the mouse Myf-6 gene

Targeting vector	#G418^r colonies	#G418^r and FIAU^r colonies	Enrichment factor	No. of recombinants	Frequency in G418^r and FIAU^r colonies	Frequency in G418^r colonies	Frequency in electroporated cells
HRCmyf6#10A	1290	663	1.95	0/424			0/0.37×10^7
HRCmyf6#14B	7720	386	20	2/386	1 of 193	1/3860	2/0.5 ×10^7
HRCmyf6#19C	13 500	540	25	34/386	1 of 11	1/397	17/1 ×10^8

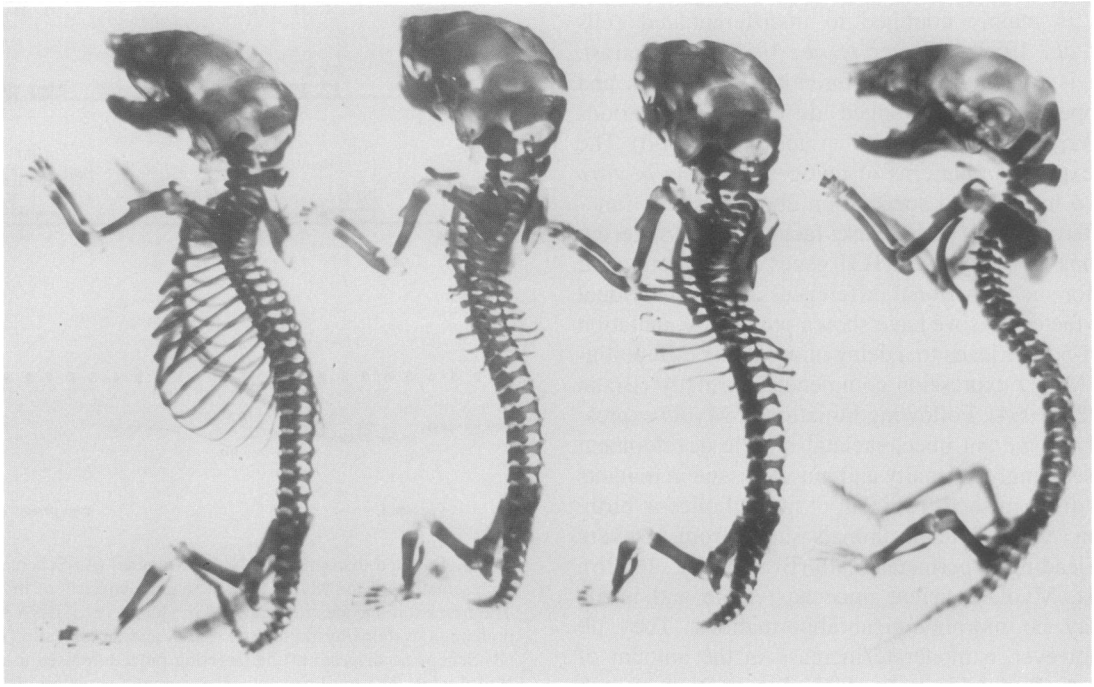

Fig. 2. Dorsal view of alizarin-stained skeletons of newborn wild-type, homozygous Myf-6 and homozygous Myf-5^{ml} mutant mice to reveal bone and cartilage structures. The animal on the left represents the wild-type, the second and third homozygous Myf-6 mutants with varying degrees of rib truncations, the fourth animal is a homozygous Myf-5^{ml} mutant included for comparison. Note the variable length of rib rudiments in Myf-6 mutants, in contrast to the consistently short ribs in Myf-5 mutants. The sternum in Myf-5 and Myf-6 homozygous mutant mice is completely ossified due to missing rib contacts. No abnormalities were detected in bones other than ribs.

orientation (Figure 1). This deletion removes the initiation codon of Myf-6 and disrupts the gene. After electroporation of J1-ES cells and double selection with G418 and FIAU, resistant colonies were picked and expanded. Thirty four homologous recombinants were identified by Southern blot analysis of DNA from 386 individual clones (Table I). Mutated ES cell clones were tested for integration of a single *neo* sequence and for the absence of genomic rearrangements downstream of the mutated exon. Five independently derived ES clones heterozygous for the Myf-6 mutation were injected into C57/BL6 or BALB/c host blastocyts to generate chimeric founders. Three clones contributed to the germline, as assessed by breeding the chimeras to BALB/c or C57/BL6 mice. Founder mice were crossed with 129SvJ mice to generate inbred lines or backcrossed with BALB/c or C57/BL6 mice. During the course of the experiments we did not find any influence of the genetic background on the phenotype generated by the Myf-6 mutation. For the present analysis heterozygous animals from F1 inbred or F1 outbred generations were crossed with each other to generate homozygous mutants.

Homozygous Myf-6 mutant mice die shortly after birth and lack the distal parts of the ribs

At birth all homozygous mutant mice were capable of spontaneous movements or reacted to mechanical stimulation, but died postnatally within a few minutes. Autopsy revealed that the rib cage was severely malformed, with only the proximal parts of the ribs next to the vertebral column normally developed. The distal parts were missing and thus no contacts with the sternum were made (Figure 2). The sternum itself was completely ossified, most likely as a secondary effect owing to the lack of sternum−rib interactions (Rugh, 1990). The observed rib phenotype in Myf-6$^-$ mice was very similar to the Myf-5 mutant mice. However, the rib stumps were generally longer in Myf-6 than in Myf-5 mutant mice, the latter consistently displaying only short rib rudiments (Braun *et al.*, 1992b). Variations in the length of ribs occurred independently of the genetic background. Alizarin red and alcian blue staining revealed no other abnormalities of the skeletal system. In particular, bones and cartilage of the limbs and the vertebral column appeared completely normal.

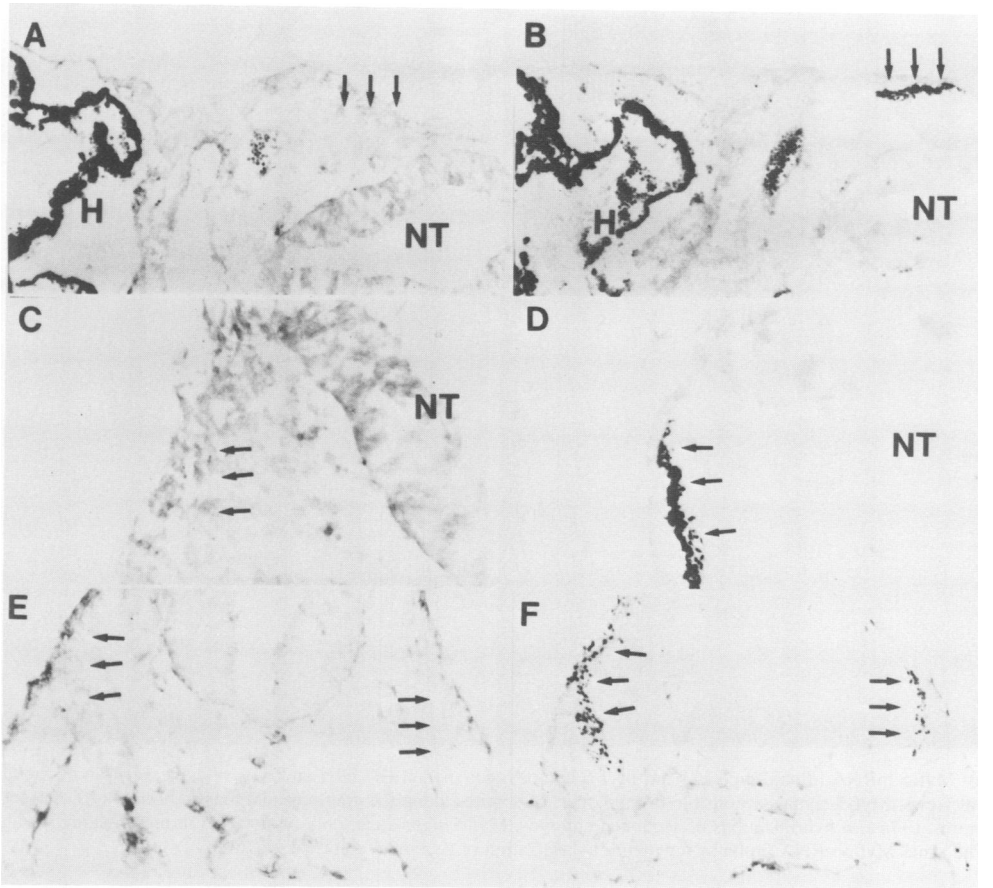

Fig. 3. Immunohistochemical staining of transverse sections of genotyped wild-type (**B**, **D** and **F**) and mutant Myf-6 (**A**, **C** and **E**) embryos at E10.0 with monoclonal antibodies against sarcomeric MyHC (A–D) and myogenin (E and F). Bound antibodies were visualized with secondary antibodies and the peroxidase reaction. In Myf-6 mutant embryos no immunoreactive cells were detectable in somites, whereas the myotome is clearly stained in wild-type embryos. Myotomes in wild-type embryos and dermomyotomal areas in Myf-5 mutant embryos are marked by arrows. Myosin staining in heart muscle tissue served as an endogenous control and indicates that only skeletal muscle cells were affected. NT, neural tube; H, heart. Magnifications 250× (A, B, E and F) and 500× (C and D).

Homozygous Myf-6 mutant mice show a delayed appearance of primary myocytes and lack Myf-5 mRNA in somites

A considerable delay in early myotome formation in Myf-5 mutants suggested that malformation of the ribs most likely originates from a disturbed interaction between the myotome and sclerotome during somite formation (Braun *et al.*, 1992b, 1994). We therefore investigated development of the myotome in Myf-6 mutant embryos from E9.5 to E11.5. Immunohistochemistry with anti-myosin heavy chain (MyHC) and anti-myogenin antibodies on comparable transverse sections of genotyped embryos revealed a lack of primary myocytes in somites of homozygous Myf-6 mutants between E9.5 and E10.5 (Figure 3). The positive staining of heart muscle tissue with anti-MyHC antibodies in the same sections served as an endogenous control and indicated that only skeletal muscle was affected. Similar results were obtained using several other muscle-specific antibodies, such as those against titin, nebulin and desmin (data not shown).

Using Myf-5 as a marker which is expressed in normal undifferentiated myoblasts, we searched for the presence of muscle precursors by whole mount hybridizations and *in situ* hybridizations in sections of wild-type and mutant embryos.

Myf-5 RNA could be easily detected in most somites of wild-type and heterozygous Myf-6 mutant embryos by whole mount hybridization (Figure 4), however, in newly formed somites Myf-5 escaped detection by this technique. In contrast, homozygous mutant Myf-6 embryos failed to show any Myf-5 hybridization signals, even in the most mature somites (Figure 4). To overcome the lower sensitivity of the whole mount hybridization procedure, we next performed *in situ* hybridizations with radioactive probes on sections from E9.5 embryos. Hybridization on serial sections also allowed a direct comparison of Myf-5 and myogenin mRNA, serving as another marker for myogenic differentiation. At E9.5 no Myf-5 mRNA was discovered in sections of mutant embryos, whereas Myf-5 RNA was strongly expressed in wild-type siblings (Figure 5). Moreover, no myogenin mRNA was observed in parallel sections, consistent with the lack of myogenin protein based on antibody staining (see Figure 3). The shape of the dermomyotome in Myf-6 mutants appeared normal, indicating that the lack of Myf-5 expression was not due to a general retardation of somite development (Figure 5B and D). Interestingly, in mutant embryos we observed no cells underneath the dermomyotome in a perpendicular orientation, which is characteristic of myogenic cells (Christ *et al.*, 1978). No myogenin expression was switched on and the differentiation process was apparently blocked, resulting in the lack of the myotome at this stage.

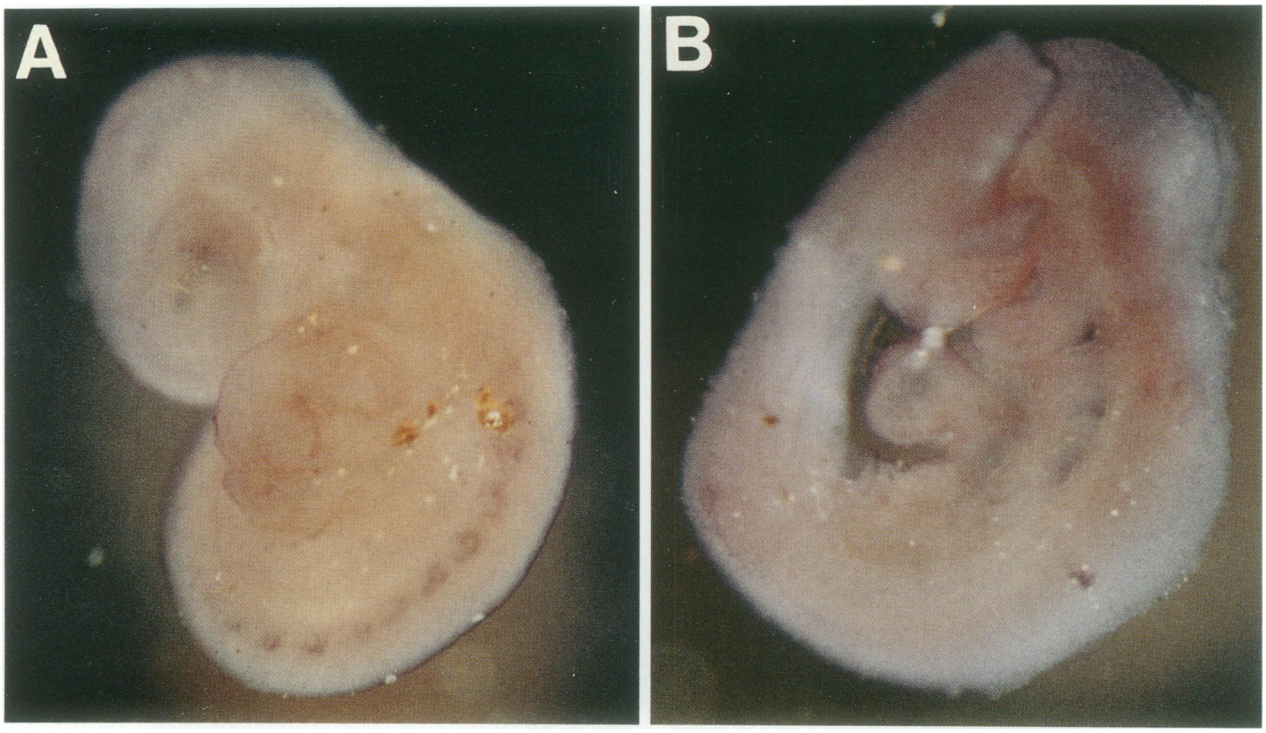

Fig. 4. Expression of Myf-5 mRNA in wild-type (**A**) and Myf-6 homozygous mutant (**B**) E9.5 embryos visualized by whole mount *in situ* hybridization. Lateral views. Myf-5 signals are visible in myotomes of somites along the cranio-caudal axis in normal (A), but not in Myf-6 mutant (B), embryos. In contrast to *in situ* hybridizations of sectioned embryos, Myf-5 signals can only be detected in more mature somites by the whole mount procedure. The same Myf-5 cRNA probe as for *in situ* hybridization of sectioned embryos was used.

In contrast, MyoD-expressing myoblasts were observed at E11.5 (data not shown), which suggests that neither Myf-6 nor Myf-5 are necessary for the development of MyoD-positive myoblasts (Braun *et al.*, 1994).

The expression of some muscle-specific genes is altered in Myf-6-deficient mice

We next asked whether Myf-5 expression was also absent during subsequent development or whether the transcription level increased. Northern blot analysis of RNA isolated from carcasses of wild-type, heterozygous and homozygous mutant Myf-6 pups revealed no Myf-5 mRNA in homozygous mutants at birth (Figure 6). Using RNase protection, however, we were able to detect a small amount of Myf-5 RNA in several homozygous mutant animals. Thus, expression of Myf-5 seems to be significantly reduced, but not completely blocked. In contrast, expression of MyoD and myogenin appeared virtually unchanged in Myf-6 mutants when compared with wild-type animals at birth. As expected, no authentic Myf-6 RNA was detected in the mutant animals. Interestingly, the amount of Myf-5 and Myf-6 RNA was also reduced in heterozygous mutants, however, no phenotypic abnormalities were discernible in these animals. Two additional faint Myf-6 bands of different size were detected in heterozygous and homozygous mutants, which most likely represent incorrectly terminated transcripts initiated either at the Myf-6 or at the *pgk* promotor.

To determine the relative expression levels of different muscle-specific mRNAs in wild-type, heterozygous and homozygous mutant mice, RNAs were isolated from carcasses of newborn mice and analyzed on Northern blots with the appropriate muscle-specific hybridization probes (Figure 6). Expression of certain muscle markers, such as MyLC1, muscle CPK and fast TnI, was slightly reduced, whereas that of γ-AChR, slow TnI and fetal MyHC was unchanged. The most drastic decrease, however, was observed for emb MyHC, which showed a moderate down-regulation in heterozygous, but a severe reduction in homozygous, Myf-6 mutants. To determine whether the down-regulation of emb MyHC was due to the lack of Myf-6 or the absence of Myf-5, we compared expression of emb MyHC in Myf-5 mutant mice with no Myf-5 but normal Myf-6 expression and Myf-6 mutant mice which lack both Myf-6 and Myf-5. RNase protection with an emb MyHC-specific cRNA probe revealed that emb MyHC was only down-regulated in homozygous Myf-6 mutant mice and not in Myf-5 homozygous mutants (Figure 7). This suggests that emb MyHC gene is dependent on the transcription factor Myf-6 or a combination of Myf-5 and Myf-6.

Deep back muscles are reduced in Myf-6 mutant mice

Data on Myf-6 expression *in vivo* and in cell culture led to the hypothesis that Myf-6 may be responsible for the generation of myocytes to form mature muscle (Emerson, 1993; Olson and Klein, 1994). Histological and immunohistochemical investigations in homozygous Myf-6 mutants do not support this hypothesis. Based on immunohistological staining with anti-MyHC antibodies, mature myotubes with regular arrays of sarcomeres were observed in mutants without significant differences from wild-type controls (Figure 8G).

To further analyze the distribution of myofibers at birth, genotyped animals were embedded in paraffin and

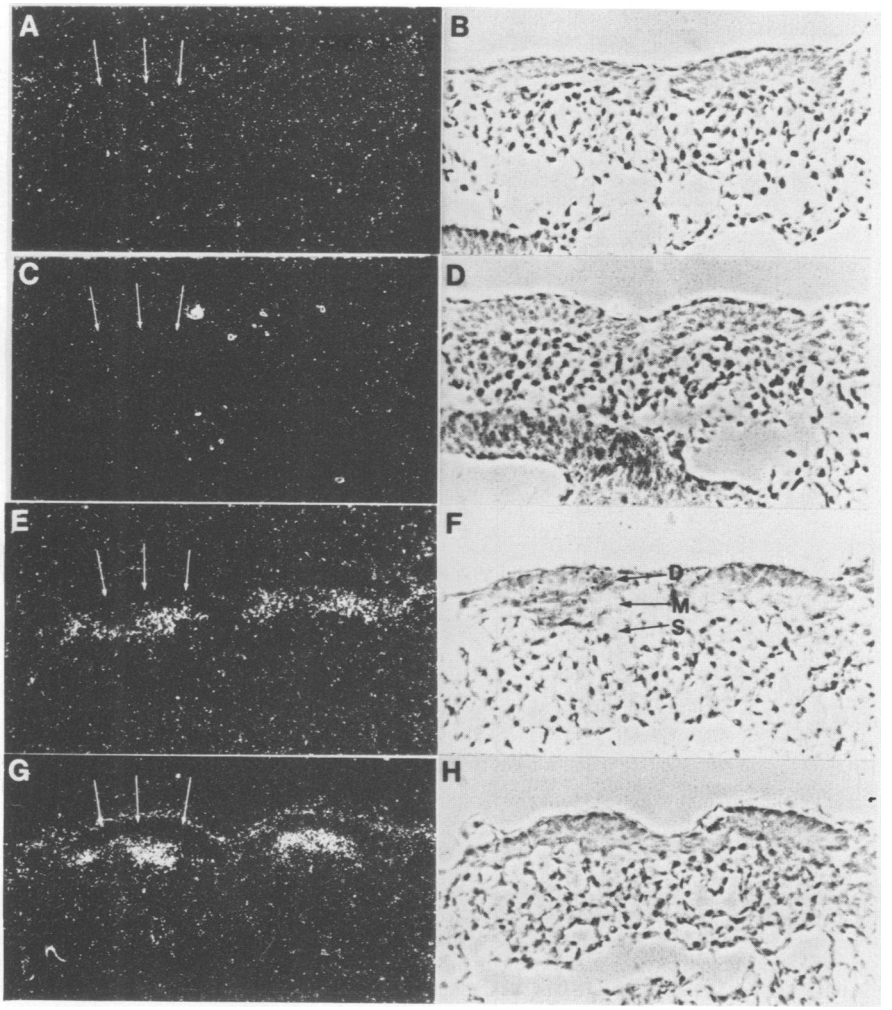

Fig. 5. Absence of Myf-5 and myogenin mRNA in genotyped homozygous Myf-6 mutants at the 22 somite stage (E9.5). *In situ* hybridization with Myf-5 (A and E) and myogenin (C and G) antisense cRNA probes to adjacent sections of trunk somites from E9.5 Myf-6 mutant (**A**−**D**) and wild-type embryos (**E**−**H**). Homozygous Myf-6 mutant embryos do not express Myf-5 or myogenin mRNA at this stage. Arrows mark positions of somites. (Left) Dark-field illumination; (right) phase contrast microscopy. The shape of the dermomyotome appears normal, but perpendicularly oriented cells underneath the dermomyotome, characteristic of myogenic cells, are not observed in the mutant embryos. D, dermomyotome; M, myotome; S, sclerotome. Magnification 500×.

transverse sections were taken along the rostro-caudal axis. Parallel sections were then stained either with hematoxylin/eosin or with the monoclonal antibody MY32, which detects fast MyHC. Using this procedure, muscles of the head, including the external eye muscles, which are derived from the prechordal plate, appeared normal (data not shown). In addition, no abnormalities in the amount and distribution of muscle fibers were recognized in the fore and hind limbs. However, we noted a significant decrease in muscle fibers of the deep axial muscles at several locations along the rostro-caudal axis (Figure 8). The remaining fibers, however, appeared normal. It seems unlikely that this reduction is caused by a differentiation block at a later stage of muscle development, since no excess of mononucleated precursor cells adjacent to differentiated muscle fibers was found by hematoxylin/eosin staining.

The Myf-5 gene is unaltered in Myf-6 mutant mice

The unexpected loss of Myf-5 expression in Myf-6 mutant mice prompted us to investigate the Myf-5 gene and its adjacent sequences very closely. In particular, all DNA sequences replaced during the mutagenesis procedure were scrutinized. Southern blot analyis of genomic DNA using several different restriction enzymes and a variety of different probes yielded no discernible difference between wild-type and Myf-6 mutants except for the intended Myf-6 mutation (data not shown). All three mouse strains which were independently derived from different ES cell clones showed the same Myf-5 down-regulation, therefore a fortuitous mutation could have been introduced only via the recombination construct itself. Although the construct was rigorously checked before use by sequence analysis of fragment ends after cloning and by restriction analysis, it was re-examined by various means. First, the gene fragments were isolated from the recombination construct, cut with various combinations of multicutters and compared with the original fragments isolated from a cosmid clone. Second, another cosmid clone which overlaps the sequences carried by the original clone was analyzed and appropriate fragments were directly compared after digestion with various enzyms. In no case was a difference

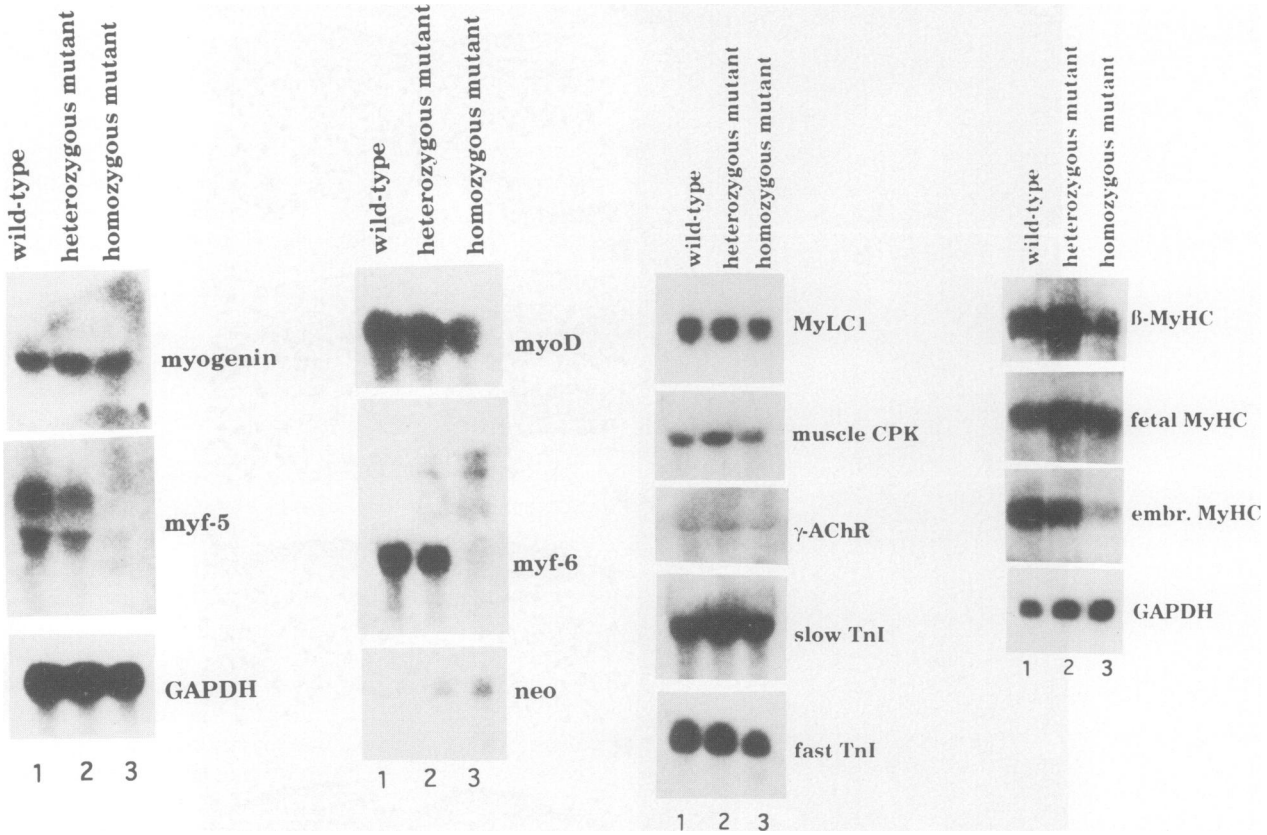

Fig. 6. Northern blot analysis of myogenic bHLH factors and skeletal muscle markers in newborn wild-type (lane1), heterozygous mutant Myf-6 (lane 2) and homozygous mutant Myf-6 mice (lane 3). Authentic Myf-6 RNA is missing in homozygous mutants. Two faint mutant transcripts were observed which most likely correspond to read-through messages initiated at the Myf-6 and the *pgk* promotor. Myf-5 RNA cannot be detected on Northern blots in homozygous mutant Myf-6 mice, but is detectable by RNase protection (not shown). Some muscle markers expressed in fast skeletal fibers were slightly reduced (fast TnI and MyLC1). The most pronounced decrease was observed for emb MyHC. This result was also confirmed by RNase protection (data not shown). The *neo* gene was transcribed in a dose-dependent manner at a low level in heterozygous and homozygous Myf-6 mutant mice.

between fragments isolated from the recombination construct, the original cosmid clone and the second cosmid clone observed (data not shown).

Furthermore, we analyzed the initiation of residual Myf-5 transcription using a RNase protection probe which covered the Myf-5 initiation start site. Although Myf-5 transcription was severely reduced in homozygous Myf-6 mutants, we were able to detect low amounts of Myf-5 RNA by this technique. All transcripts were faithfully initiated at the Myf-5 promotor (Figure 9), indicating that no rearrangement had occurred in the promotor region and that the Myf-5 sequence itself was intact.

Discussion

Control of myogenesis during development is achieved by an intricate network of different factors interacting with each other in positive and negative regulatory circuits. The only gene of the myogenic control factor family which has not been mutated in mice is Myf-6. To elucidate its role in myogenesis we have generated mouse strains with a targeted mutation in the Myf-6 gene which unexpectedly also down-regulated Myf-5 gene expression. Therefore, these mice represent a double knock-out model and exhibit the characteristics of the Myf-5 mutation as one aspect of their phenotype.

Myf-5 down-regulation in Myf-6 mutant mice may be explained in two ways. First, the Myf-6 gene mutation may affect Myf-5 expression in *cis*, as both genes are closely linked and only 9 kb apart on mouse chromosome 10. Second, Myf-6 may be required to regulate the Myf-5 gene in *trans*.

The simplest explanation of a mutation in a Myf-5 regulatory element appears unlikely, since only the exon sequence of the Myf-6 gene has been deleted. Rearrangements or deletions above the level of point mutations in the vector have also been excluded. The occurrence of a fortuitous mutation in the Myf-5 gene outside the sequences which were replaced by homologous recombination is also unlikely, since three independently derived ES cell clones were used to generate Myf-6 mutant mice, with the same phenotype in all strains. However, it is possible that insertion of the *pgk–neo* genes may interfere with a remote Myf-5 activating element which is located outside of both genes. In fact, a promotor/enhancer competition model has been previously proposed for a mutation of the locus control region in the globin cluster (Kim *et al.*, 1992; Fiering *et al.*, 1993). Alternatively, the spacing of regulatory elements in the Myf-6/Myf-5 locus may be important. The integration of the *pgk–neo* cassette in combination with a small deletion in the first exon of the Myf-6 gene results in introduction of

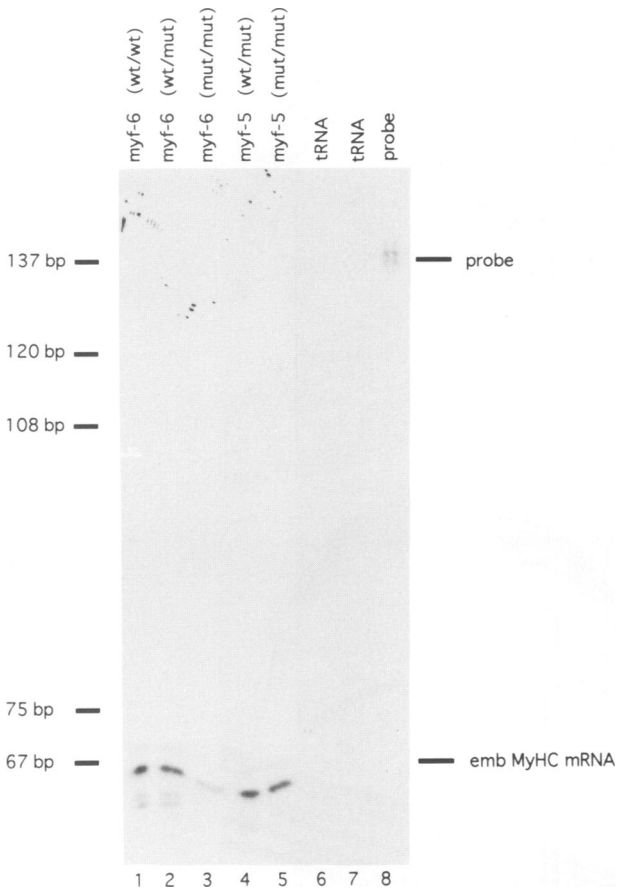

Fig. 7. RNase protection of emb MyHC mRNA in wild-type, Myf-5 mutant and Myf-6 mutant embryos. Lane 1, wild-type RNA; lane 2, heterozygous Myf-6 RNA; lane 3, homozygous Myf-6 RNA; lane 4, heterozygous Myf-5 RNA; lane 5, homozygous Myf-5 RNA; lanes 6 and 7, tRNA; lane 8, undigested probe. Emb MyHC is reduced in homozygous mutant Myf-6 mutants, but not in wild-type and Myf-5 mutant embryos. Emb MyHC RNA was detected with a 137 nt probe including 70 nt vector sequences. The predicted 67 nt MyHC fragment was derived from the 3′-untranslated region of the mouse cDNA. RNA was isolated from wild-type, heterozygous and homozygous mutant Myf-5 and Myf-6 newborn animals and checked for integrity and equal loading by ethidium bromide staining of ribosomal RNA before hybridization.

an additional 1.6 kb DNA sequence between a potential upstream regulatory element and the Myf-5 gene.

In addition, interference by the integrated *pgk* promotor with the Myf-5 promotor is feasible. However, such a mechanism would have to be position-dependent, as insertion of the same *pgk−neo* cassette in the Myf-5 gene did not impair transcriptional initiation of the Myf-5 promotor (Braun *et al.*, 1992b; Braun and Arnold, 1994). Interference of large aberrant Myf-6 transcripts observed in Myf-6 mutant mice with the constitution of the transcriptional initiation complex on the Myf-5 promotor seems unlikely, since the detected mutant transcripts are ~3.5 and 3.7 kb in size, which is not sufficient to extend into the Myf-5 promotor region.

Although the status of the chromatin structure of the mutant Myf-6 locus is unknown, it cannot be excluded that opening of the chromatin in the first exon of the Myf-6 gene caused by the active *pgk* promotor may affect the chromatin structure of the Myf-5 gene and alter its accessibility for transcription factors.

Myf-5 down-regulation in *trans* caused by the lack of Myf-6 may be less likely. Such a mechanism would not be compatible with the temporal activation pattern of the Myf-6 gene following Myf-5 expression, as previously defined by *in situ* hybridization (Bober *et al.*, 1991; Hinterberger, *et al.*, 1991). It should be mentioned, however, that Myf-6 transcripts can be detected in immature somites and even in unsegmented mesoderm by reverse transcription−polymerase chain reaction, indicating that low level Myf-6 transcription occurs much earlier than previously noticed (data not shown). This expression is very low and can be detected only after 40 rounds of amplification. Whether these minute quantities of transcripts are sufficient to account for biological activity cannot be assessed in this way. Whatever the mechanism for Myf-5 down-regulation may be, our observations emphasize the importance of a rigorous analysis of gene activities adjacent to targeted gene loci. In particular, knock-out experiments in complex gene clusters in which regulatory mechanisms are unknown or only partially understood have to be considered with care. Rescue experiments or the introduction of point mutations may be mandatory in such cases. A critical problem remains when neighboring genes or control regions are unknown.

The lack of Myf-5 transcription in somites of Myf-6 mutant mice explains the malformation of the ribs, already reported for homozygous Myf-5 mutant mice (Braun *et al.*, 1992b). As in Myf-5-deficient mice, there are probably no myoblasts and primary myocytes in the myotome of E8−E11 Myf-6 mutant embryos which could stimulate the outgrowth of ribs from the lateral sclerotome (Holtzer, 1968; Hall, 1977). The larger size of rib rudiments in many Myf-6 mutants may be due to low residual Myf-5 expression supplying a weak signal which results in the elongation of rib anlagen under these conditions.

A large body of evidence exists that myogenic bHLH proteins may act as transcription factors to activate muscle-specific genes (Braun *et al.*, 1990b, 1992a; Davis *et al.*, 1990; Brennan *et al.*, 1991). Inactivation of myogenic factors, however, either led to the total absence of myoblasts and myocytes in early somites (Myf-5) and later during development (Myf-5 and MyoD) or to a partial block of differentiation (Braun *et al.* 1992b, 1994; Rudnicki *et al.*, 1992, 1993; Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). The high level of Myf-6 expression in mature muscle fibers, as well as transactivation experiments which indicated its critical role in the activation of certain muscle genes, made Myf-6 a particularly interesting candidate for mediating transactivation of certain muscle genes. The down-regulation of emb MyHC, which is not observed in Myf-5 mutant animals, may be one example of the dependence of structural muscle genes on distinct transcription factors. It is interesting to note that expression of most muscle-specific genes was not affected by the lack of Myf-6. Overlapping functions between other myogenic regulatory factors or the MEF-2 family of transcription factors may rescue the expression of these muscle genes. Although emb MyHC expression is normal in Myf-5 mutant embryos, it cannot be excluded that the down-regulation of emb MyHC expression is caused by the lack of both Myf-6 and Myf-5 and not by the absence of Myf-6 alone. Since Myf-5 is severely reduced in Myf-6 mutant

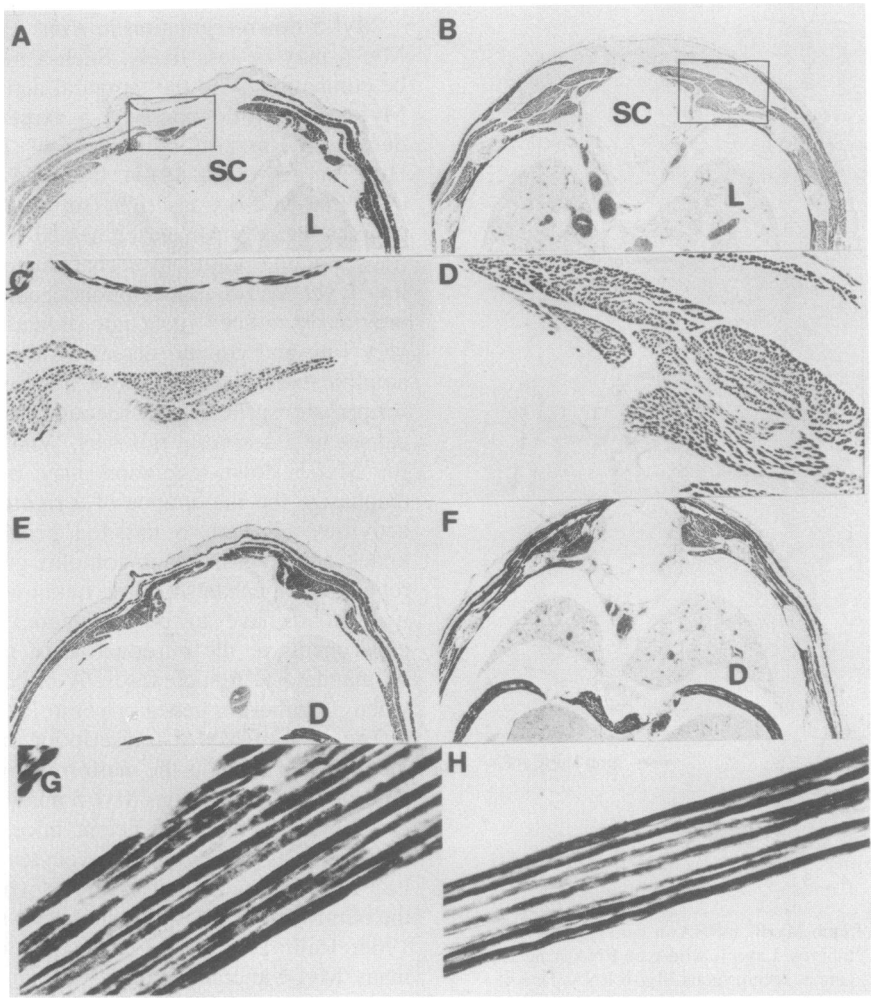

Fig. 8. Muscle phenotype of Myf-6-deficient mice. Immunohistochemical staining for MyHC on sections of homozygous mutant Myf-6 (**A**, **C**, **E** and **G**) and wild-type (**B**, **D**, **F** and **H**) littermates at the newborn stage. Deep axial muscles in the thoracic region appear reduced when stained with anti-fast MyHC antibody. Cross-sections of mutant and wild-type animals at comparable positions in the body axis were analyzed. The areas framed in (A) and (B) are enlarged in (C) and (D). (A) and (B) are from a high thoracic level, where the fat pad in the back of newborn mice is still visible. (E) and (F) are from a low thoracic level, where the diaphragm is already detectable. The diaphragm, superficial body wall musculature and limb muscles were not reduced in size. Cross-striations in mature secondary myotubes are visible in homozygous mutant (G) and wild-type (H) animals at high magnification. SC, spinal cord; L, lung; D, diaphragm. Magnifications 62.5× (A, B, E and F), 250× (C and D) and 1000× (G and H).

mice, we were unable to distinguish between these two possibilities.

Immunohistochemical staining of transverse sections along the rostro-caudal axis of Myf-6 mutant mice at birth with an anti-fast MyHC antibody revealed a severely reduced number of muscle fibers in the deep muscles of the back, but normal muscle masses in the limbs. Interestingly, Nabeshima and co-workers (1993) reported a complementary distribution of residual muscle cell differentiation in myogenin knock-out mice, e.g. more residual differentiation in axial than in limb muscles. In contrast to the observations in myogenin mutant mice, we did not find a surplus of mononucleated cells amongst differentiated muscle fibers. Therefore, a simple differentiation block caused by the absence of Myf-6 appears unlikely. Our hypothesis is that Myf-6 alone or together with Myf-5 is more important in determining the number of cells in axial muscles, which originate from the myotome, than of limb muscles, which come from the dermomyotome.

However, it also seems possible that alterations in deep axial muscles are caused by the lack of some muscle attachment sites due to malformation of the ribs, with subsequent atrophy of the corresponding muscles. Direct comparison of individual muscles between the wild-type and mutants will be necessary to answer this question.

Critical differences between axial and limb muscle development have been documented: axial muscle formation depends on the neural tube, whereas limb and superficial body wall musculature develops independently of neural influence (Christ *et al.*, 1992; Rong *et al.*, 1992; Bober *et al.*, 1994a). In contrast, limb muscles require Pax-3 to form normally (Bober *et al.*, 1994b; Goulding *et al.*, 1994; Williams and Ohrdal, 1994). Myf-6 expression seems to be limited to precursors of axial muscles and is not detectable at high levels during the early stages of limb muscle development (Bober *et al.*, 1992). Taken together, it is reasonable to assume that development of superficial body wall and limb muscles is less dependent on Myf-6 than that of axial muscles.

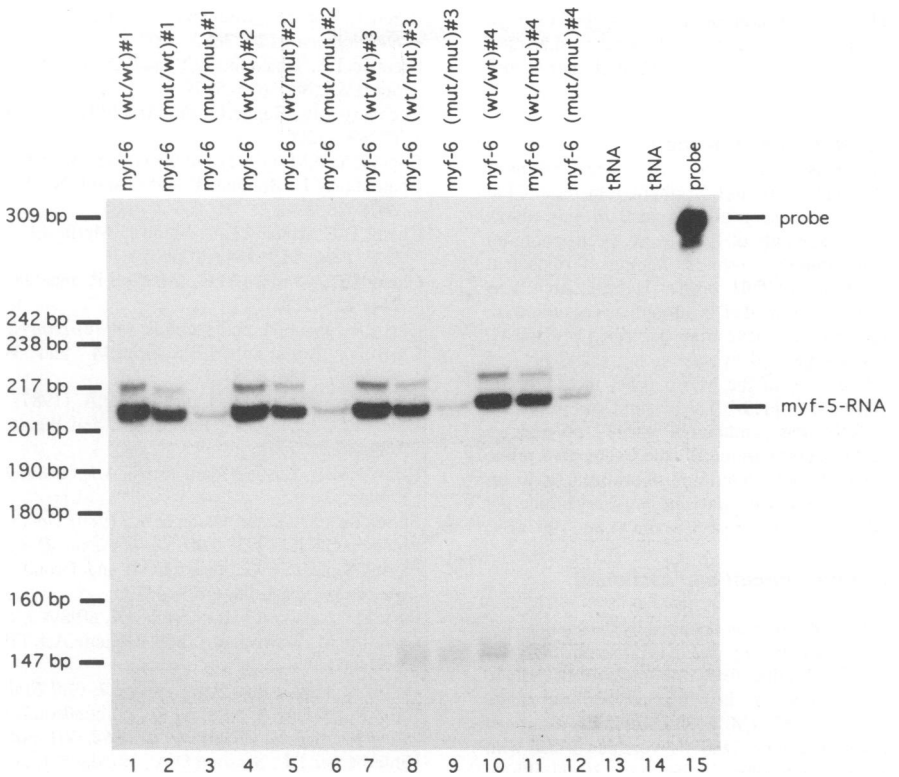

Fig. 9. RNase protection analysis of Myf-5 expression in wild-type, heterozygous and homozygous mutant Myf-6 mice. RNAs of 12 different siblings from one litter were analyzed. Lanes 1, 4, 7 and 10, wild-type RNA; lanes 2, 5, 8 and 11, heterozygous Myf-6 RNA; lanes 3, 6, 9 and 12, homozygous Myf-6 RNA; lanes 13 and 14, tRNA; lane 15, undigested probe. Myf-5 RNA was detected with a 312 nt RNA probe that included 92 nt vector sequences. The predicted 205 nt fragment was derived from the first exon of the Myf-5 gene, allowing the determination of correct transcriptional initiation. Transcription of the Myf-5 gene is slightly reduced in heterozygous animals and strongly reduced in homozygous mutant Myf-6 animals. Note the correct initiation of transcription in homozygous mutant mice. The presence of two bands in the protection assay is due to probe 'breathing', probably caused by a high AT content of the probe. Both bands were equally reduced in Myf-6 mutant mice.

The muscle phenotype of Myf-6$^-$ mutants which express only small residual amounts of Myf-5 mRNA is relatively mild. This is in marked contrast to homozygous mutant Myf-5 and MyoD mice, which lack all skeletal muscle myocytes and, presumably, myoblasts (Rudnicki *et al.*, 1993). These findings support the view that functional complementation groups may exist within the family of myogenic factors (Braun and Arnold, 1994). According to this idea, major consequences for the development of the muscle cell lineage will arise only when all members of a group are inactivated. Thus, Myf-5 and Myf-6, unlike Myf-5 and MyoD, do not complement each other, indicating their different roles in the process of myogenic differentiation.

The generation of mice deficient in the other three members of the MyoD family has already yielded important insights into individual and redundant functions of these factors (Braun *et al.*, 1992b; Rudnicki *et al.*, 1992, 1993; Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). In this report we provide evidence that Myf-6 may be the critical factor controlling transcription of the emb MyHC gene and for generation of the full deep musculature of the back. Due to the perinatal lethality of the Myf-6 knock-out mutation, it was not possible to test functions of Myf-6 in the adult animal. Experiments to introduce a conditional mutation in the Myf-6 gene allowing investigation of these functions are underway.

Materials and methods

Construction of the targeting vector, electroporation of ES cells and generation of mice

The Myf-6 gene was isolated from a cosmid library made from J1-ES cell DNA (kindly provided by John S.Mudgett) by screening with a mouse Myf-6 cDNA probe (Bober *et al.*, 1991). Two different cosmid clones were identified and further characterized by restriction enzyme analysis and Southern blotting. A 9 kb *Kpn*I fragment was identified which contains parts of exons I, II and III of the Myf-6 gene, the intergenic region between the Myf-6 and Myf-5 gene and exon I of the Myf-5 gene. The *Kpn*I fragment was subcloned into the *Kpn*I site of recombination vector pPNT, yielding pPNT-Kpn. A 4 kb *Xba*I fragment was identified within the cosmid which carries most of the coding information of the Myf-6 gene and was subcloned into the *Xba*I site of pKS. From this plasmid a 2.5 kb *Sal*I fragment was released and subcloned into the *Xho*I site of pPNT-Kpn, resulting in the final recombination vector HRCmyf6#19C.

Embryonic stem cell line J1 was grown on embryonic feeder cells (EF cells) as described (Braun *et al.*, 1992b). Electroporation, selection and analysis of ES clones were all done as described previously (Braun *et al.*, 1992b). Five randomly chosen recombined ES cell clones were injected into C57Bl6 or BALB/c mouse blastocysts. Three clones contributed to the germline and were independently used to establish mouse strains.

Anatomical analysis and immunohistochemical staining

For bone and cartilage staining, newborn animals were sacrificed and skinned. Staining was performed as described (McLeod, 1980).

For immunohistochemical staining, newborn mice were sacrificed and fixed in formalin for several days before embedding in paraffin wax (Bancroft and Stevens, 1990). Serial sections were taken at 7 μm. Before staining with monoclonal antibody MY32 against fast MyHC (Sigma),

sections were dewaxed and fixed with acetone. Bound antibodies were visualized with a Vectastain elite kit using diaminobenzidine as substrate. Hematoxylin/eosin staining was performed as described (Bancroft and Stevens, 1990).

Northern blot analysis and RNase protection

Isolation of total RNA from carcasses of newborn mice, denaturation of RNA with glyoxal, gel electrophoresis and Northern transfer on Pal Biodyne A membrane were done according to standard procedures (Sambrook *et al.*, 1989). Equal amounts of RNA were electrophoresed in each lane, as assessed by ethidium bromide staining of ribosomal RNA and hybridization with a GAPDH probe. Hybridization was performed with ^{32}P-labeled random primed cDNA fragments as indicated. Hybridization and washing conditions have been described previously. An antisense RNA probe was generated by subcloning a 200 bp *Pst*I fragment which spans the first exon of the Myf-5 gene, including the *neo* gene insertion site, into the vector pKSII. The plasmid was linearized with *Hin*dIII and antisense RNA was synthesized with T7 polymerase in the presence of [α-^{32}P]UTP (3000 Ci/mmol). The radioactive probe was purified on a polyacrylamide gel, eluted and hybridized to 50 μg total RNA. RNase digestion, probe purification and polyacrylamide gel electrophoresis have been described previously (Braun *et al.*, 1992a).

In situ hybridization on whole mount and sectioned embryos

Material for genotyping of the embryos was obtained by dissecting yolk sac tissue from the embryo proper. For *in situ* hybridization, embryos were washed in phosphate-buffered saline, fixed in 4% paraformaldehyde for 12 h and treated with 0.5 M sucrose in phosphate-buffered saline overnight. Embryos were frozen in OCT (Miles Scientific) and sectioned on a cryostat. Prehybridizations and hybridizations were performed with [^{35}S]UTP-labeled cRNA probes as described (Bober *et al.*, 1991). Whole mount hybridizations were performed with digoxigenin-labeled cRNA probes as described by Wilkinson (1992).

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