MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle

Boris Kablar1, Kirsten Krastel1, Chuyan Ying1, Atsushi Asakura2, Stephen J. Tapscott2 and Michael A. Rudnicki1,3

1Institute for Molecular Biology and Biotechnology, Cancer Research Group, McMaster University, Hamilton, Ontario, Canada L8S 4K1
2Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104, USA
*Author for correspondence (e-mail: rudnicki@mcmaster.ca)

SUMMARY

The myogenic progenitors of epaxial (paraspinal and intercostal) and hypaxial (limb and abdominal wall) musculature are believed to originate in dorsal-medial and ventral-lateral domains, respectively, of the developing somite. To investigate the hypothesis that Myf-5 and MyoD have different roles in the development of epaxial and hypaxial musculature, we further characterized myogenesis in Myf-5−/− and MyoD−/− deficient embryos by several approaches. We examined expression of a MyoD-lacZ transgene in Myf-5 and MyoD mutant embryos to characterize the temporal-spatial patterns of myogenesis in mutant embryos. In addition, we performed immunohistochemistry on sectioned Myf-5 and MyoD mutant embryos with antibodies reactive with desmin, nestin, myosin heavy chain, sarcomeric actin, Myf-5, MyoD and myogenin. While MyoD−/− embryos displayed normal development of paraspinal and intercostal muscles in the body proper, muscle development in limb buds and brachial arches was delayed by about 2.5 days. By contrast, Myf-5−/− embryos displayed normal muscle development in limb buds and brachial arches, and markedly delayed development of paraspinal and intercostal muscles. Although MyoD mutant embryos exhibited delayed development of limb musculature, normal migration of Pax-3-expressing cells into the limb buds and normal subsequent induction of Myf-5 in myogenic precursors was observed. These results suggest that Myf-5 expression in the limb is insufficient for the normal progression of myogenic development. Taken together, these observations strongly support the hypothesis that Myf-5 and MyoD play unique roles in the development of epaxial and hypaxial muscle, respectively.

Key words: epaxial, hypaxial, Myf-5, MyoD, muscle, mouse, limb, epaxial muscle, hypaxial muscle, somite

INTRODUCTION

The myogenic regulatory factors (MRFs), a group of basic helix-loop-helix (bHLH) transcription factors consisting of MyoD, myogenin, Myf-5 and MRF4, play important regulatory functions in the skeletal-muscle differentiation program (Weintraub et al., 1991). The roles played by the MRFs during embryogenesis have been greatly elucidated by gene targeting in mice. The introduction of null mutations in Myf-5, MyoD, myogenin and MRF4 into the germline of mice has revealed the hierarchical relationships existing among the MRFs and established that functional redundancy is a feature of the MRF regulatory network (Rudnicki et al., 1992, 1993; Braun et al., 1992; Hasty et al., 1993; Nabeshima et al., 1993; Patapoutian et al., 1995; Zhang et al., 1995). The MRF family can be divided into two functional groups. The primary MRFs, MyoD and Myf-5, appear to be required for the determination of skeletal myoblasts. The secondary MRFs, myogenin and MRF4, act later in the program likely as differentiation factors (reviewed by Rudnicki and Jaenisch, 1995; Megeney and Rudnicki, 1995).

Vertebrate skeletal muscle is derived from multipotential cells in the prechordal and somitic mesoderm that give rise to committed myogenic precursor cells which become the skeletal muscle of the head, trunk and limbs (reviewed by Hauschka, 1994). Somites arise as condensations of paraxial mesoderm, which form epithelial spheres on either side of the neural tube. The ventral portion of the somite partitions into a structure called the sclerotome which gives rise to the cartilage and bone of the trunk. The dorsal-lateral portion of the somite gives rise to the dermamyotome which partitions into the dermatome and myotome. The dermamyotome is the source of all skeletal myogenic precursor cells within the segmented region of the developing embryo and each somite gives rise to between 30 and 100 migratory myogenic precursor cells (Orudahl and Le Douarin, 1992; reviewed by Hauschka, 1994; Christ and Orudahl, 1995).

Epaxial (paraspinal and intercostal) and hypaxial (limb and abdominal wall) musculature are believed to have distinct origins suggesting that they may be formed by different myogenic lineages. For example, epaxial musculature is formed by myogenic precursors that are derived from the dorsal-medial portion of the dermamyotome, whereas hypaxial musculature is formed by myogenic precursors that are derived

Vertebrate skeletal muscle is derived from multipotential...
from the ventral-lateral portion of the dermamyotome (Ordahl and Le Douarin, 1992; reviewed by Hauschka, 1994; Christ and Ordahl, 1995). Myf-5 is initially expressed in cells derived from the dorsal-medial portion of the dermamyotome that gives rise to the precursors that form epaxial muscles, and MyoD is initially expressed in cells derived from the ventral-lateral portion of the dermamyotome that gives rise to the precursors that form hypaxial muscles. Taken together, these observations suggest the hypothesis that Myf-5 and MyoD have in part, unique roles in the development of epaxial and hypaxial musculature respectively (Smith et al., 1994; Rudnicki and Jaenisch, 1994; Cosu et al., 1996b).

To investigate the hypothesis that Myf-5 and MyoD have different roles in the development of epaxial and hypaxial musculature, we performed an analysis of the temporal-spatial pattern of myogenesis during the development of Myf-5 and MyoD mutant embryos. In Myf-5-deficient embryos, we observed delayed development of epaxial musculature, but surprisingly, we observed normal development of hypaxial musculature. Conversely, in MyoD mutant embryos, we observed delayed development of hypaxial musculature and normal development of epaxial musculature. Taken together, these results support the hypothesis that Myf-5 has a primary function in the regulation of epaxial muscle development, whereas MyoD has a primary function in the regulation of hypaxial muscle development.

**MATERIALS AND METHODS**

**Interbreeding and collection of embryos**

The MyoD-lacZ (MD6.0-lacZ) mice carry a transgene in which 6.0 kb of MyoD sequence upstream from the transcription start site is linked to the bacterial lacZ gene (Asakura et al., 1995). The MD6.0- lacZ transgenic mice were first bred with MyoD-/- mice (Rudnicki et al., 1992) to generate MyoD+/−:lacZ mice which were subsequently interbred with MyoD-/- mice (Braun et al., 1992) to generate MyoD-/- :MyoD+/−:lacZ mice. Myf-5+/−:MyoD+/−:lacZ mice were interbred to obtain embryos of 9 different genotypes. Embryos and the fetal portion of the placenta were collected by Cesarean section between 10.5 and 13.5 days post coitum (dpc), embryos were fixed and stained, and DNA isolated from placentas as described below.

**Genotyping and β-galactosidase staining of embryos**

Genomic DNA was isolated from the fetal placenta using the procedure of Laird et al. (1991). Embryos were genotyped by Southern analysis (Sambrook et al., 1989) of placental DNA using Myf-5-, MyoD-, and β-Gal-specific probes as described previously (Braun et al., 1992; Rudnicki et al., 1992). β-galactosidase expression was detected as described by Asakura et al. (1995). Embryos were fixed 1-2 hours (2% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer pH 7.4 and 2 mM MgCl₂) washes twice for 30 minutes in solution A (0.1 M phosphate buffer pH 7.4, 2 mM MgCl₂, 0.1% sodium deoxycholate, 0.2% Nonidet P-40) and then washed twice, for 30 minutes in solution B (0.1 M phosphate buffer pH 7.4, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.2% Nonidet P-40). β-galactosidase was detected by overnight incubation at 37°C in 1.0 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide. Embryos were washed twice for 1 hour at room temperature in phosphate-buffered saline (PBS), and then post-fixed overnight, as described above. Embryos were washed twice for 1 hour at room temperature in PBS and stored at 4°C in 70% ethanol. At least three embryos of each genotype, containing two alleles of the lacZ transgene, were derived and characterized. Whole embryos were photographed in 70% ethanol using a Zeiss dissecting microscope.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (Rudnicki et al., 1993) on paraffin-embedded 4 μm sections with mouse monoclonal anti-desmin antibody D33 (DAKO, Glostorp, Denmark), mouse monoclonal anti-nestin antibody Rat-401 (Developmental Studies Hybridoma Bank), mouse monoclonal anti-sarcomeric actin antibody SC5 (Sigma), mouse monoclonal anti-myosin heavy chain antibody MF20 (Bader et al., 1982), rabbit polyclonal anti-MyoD antibody M-318 (Santa Cruz), rabbit polyclonal anti-Myf-5 antibody C-20 (Santa Cruz) and rabbit polyclonal anti-myogenin antibody m225 (Santa Cruz). Primary antibodies were diluted as follows: 1:100, 1:4, 1:600, 1:10, 1:50, 1:100 and 1:20, respectively.

**Whole-mount in situ hybridization**

Expression of the Pax-3 gene was analyzed by in situ hybridization using a 520 bp HindIII/Pst fragment of the cDNA encoding the 3’ part of the paired-type homeodomain and most of the carboxyterminus (Goulding et al., 1994). Mouse embryos were collected and treated as described by Wilkinson (1992). Synthesis of sense and antisense digoxigenin-labeled RNA probes was performed using a DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer’s instructions. Whole embryos were photographed in 70% ethanol using a Zeiss dissecting microscope.

**RESULTS**

**Expression of a MyoD-lacZ transgene in Myf-5- and MyoD mutant embryos**

To facilitate a temporal-spatial characterization of muscle development in mutant embryos, we bred mice carrying a MyoD-lacZ transgene (Asakura et al., 1995) with Myf-5- and MyoD mutant mice. The MyoD-lacZ transgene (MD6.0-lacZ) contains 6 kb of MyoD upstream regulatory sequences including a potent muscle-specific enhancer located about 5 kb upstream from the MyoD transcription start site (Tapscott et al., 1992; Asakura et al., 1995). Expression of the MD6.0-lacZ transgene appears to closely follow myogenic development and is expressed in differentiated embryonic myocytes (Asakura et al., 1995). Importantly, the MD6.0-lacZ transgene is not expressed in myogenic progenitor cells as is 258/lacZ containing the −20 kb MyoD enhancer linked to 2.5 kb of MyoD promoter sequences and lacZ (Goldhamer et al., 1995; K. Krastel and M. Rudnicki, unpublished). Therefore, the MD6.0- lacZ transgene functions as an in vivo muscle-specific marker and, as such, is useful for analyzing the developmental progression of the skeletal-muscle differentiation program during embryogenesis. Treated matings were performed with heterozygous mice containing the lacZ transgene to generate mutant embryos at different developmental stages.

As previously described, MD6.0-lacZ transgenic wild-type embryos (containing functional Myf-5 and MyoD genes), first exhibit lacZ expression at 10.5 dpc in myotomal regions rostral to the forelimb buds in the cervical region (Fig. 1A, arrow). By 11.5 dpc, myogenic development was underway along the entire rostral-caudal axis of the embryo as evidenced by lacZ expression in myotomes (Fig. 1D, arrows). Expression in the forelimb buds (arrowhead) and brachial arches was first observed at 11.5 dpc (Fig. 1D and 2D) and, in the hindlimbs, by 12.5 dpc (Fig. 1G). By 13.5 dpc, individual muscle masses
were readily discernible due to abundant lacZ expression in virtually all skeletal muscle (Fig. 1J). Ectopic expression of the MD6.0-lacZ transgene was observed in the neural tube and spleen of all 10.5 and 11.5 dpc embryos (Fig. 1) as described elsewhere (Asakura et al., 1995).

The distribution of myocytes in anterior somites of 10.5 dpc Myf-5$^{-/-}$ embryos, as revealed by MD6.0-lacZ transgene expression, appeared similar to wild-type embryos suggesting that cervical myogenic development is not Myf-5 dependent (compare Fig. 1A,C). However, by 11.5 dpc Myf-5$^{-/-}$ embryos exhibited little or no development of paraspinal and intercostal muscles in the trunk proper with virtually no myotomal lacZ expression detectable below the cervical level suggesting that the myotomal myogenic program in the trunk is Myf-5 dependent (compare Fig. 1D,F). Examination of 12.5 dpc Myf-5$^{-/-}$ embryos revealed the presence of primitive trunk musculature relative to wild type with only small rudimentary muscles apparent along the rostral caudal axis (arrow in Fig. 1F). This reduction in the development of trunk musculature in Myf-5$^{-/-}$ embryos was less apparent at 13.5 dpc (Fig. 1L). Unexpectedly, muscle development in the forelimbs, hindlimbs, brachial arches and cervical regions of 11.5, 12.5 and 13.5 dpc Myf-5$^{-/-}$ embryos, as revealed by lacZ expression, was virtually identical to the pattern observed in wild-type embryos (compare Fig. 1G to 1I, and 1J to 1L; Fig 2D to 2F, 2G to 2I, and 2J to 2L).

In contrast to Myf-5$^{-/-}$ embryos, examination of MyoD$^{+/+}$ embryos revealed normal development of paraspinal and intercostal musculature in the trunk proper. The MD6.0-lacZ transgene was expressed normally in the cervical regions of the trunk above the level of the forelimb at 10.5 dpc (Fig. 1B, arrow). In addition, from 11.5 onwards (Fig. 1E,H,K), the development of trunk musculature appeared normal except that the ventral spread of myogenesis in thoracic regions of the trunk was delayed relative to wild type (compare Fig. 1G,H).

Closer examination of MD6.0-lacZ expression in MyoD$^{-/-}$ embryos revealed notable differences from wild-type and Myf-5$^{-/-}$ genotypes with a marked delay evident in the onset of myogenesis in forelimbs, hindlimbs and brachial arches. At 11.5 dpc, the first appearance of musculature was observed in the forelimbs of wild-type and Myf-5$^{-/-}$ embryos (see Figs 1D,F, 2D,F). However, 11.5 dpc MyoD$^{+/+}$ embryos completely lacked any lacZ expression in forelimbs (arrowhead in Figs 1E, 2E). Both wild-type and Myf-5$^{-/-}$ 12.5 dpc embryos had welldescribed muscle anlagen in forelimbs, hindlimbs and brachial arches as evidenced by lacZ expression (Figs 1G, 2G, 1I, 2I). By contrast, MyoD$^{-/-}$ 12.5 dpc embryos continued to exhibit delayed myogenic development in these areas (Figs 1H, 2H). Moreover, expression in the shoulder muscles of MyoD$^{-/-}$ embryos appeared markedly reduced compared to wild type at day 12.5, suggesting that muscle development was delayed

---

**Fig. 1.** Expression of MD6.0-lacZ in Myf-5 and MyoD mutant embryos reveals differences in myogenic development. At 10.5 dpc, similar expression of MD6.0-lacZ was observed in cervical regions (green arrow) in wild-type (A), MyoD$^{+/+}$ (B) and Myf-5$^{-/-}$ (C) embryos. By 11.5 dpc, wild-type embryos (D) displayed progressing myotomal myogenic differentiation in the trunk (green arrows) and the first detectable myogenic differentiation in the forelimb bud (yellow arrowhead). While MyoD$^{+/+}$ 11.5 dpc (E) and 12.5 dpc (H) embryos displayed normal muscle development in the body proper (green arrows), no lacZ expression was detectable in the muscle anlagen of the forelimbs (yellow arrowhead). Also note the reduced expression of lacZ in the brachial arches in 11.5 and 12.5 dpc MyoD$^{+/+}$ embryos (red arrowheads). By contrast, Myf-5$^{-/-}$ 11.5 dpc (F) and 12.5 dpc (I) embryos displayed delayed muscle development in the body proper (green arrows) and normal expression of lacZ the muscle anlagen of the forelimbs (yellow arrowhead). By 13.5 dpc, lacZ expression in the limb buds (yellow arrowheads) was similar between wild-type (J), MyoD$^{+/+}$ (K) and Myf-5$^{-/-}$ (L) embryos. Note the presence of ectopic transgene expression in the spleen and neural tube (Asakura et al., 1995).
within the entire limb girdle (Figs 1H, 2H). By 13.5 dpc, the limbs, and brachial arches of \textit{MyoD}⁻/⁻ embryos contained \textit{lacZ}-expressing muscles but of smaller size of those observed in wild-type embryos (compare Figs 1J and K, 2J and K).

The expression pattern of \textit{MD6.0-lacZ} transgene in \textit{Myf-5} and \textit{MyoD} mutant embryos suggested that the progression of myogenesis differs in anatomically distinct regions between the different genotypes. Development of trunk musculature has been previously reported to be delayed in embryos lacking \textit{Myf-5} until the onset of \textit{MyoD} expression (Braun et al., 1994). However, our results revealed that \textit{Myf-5} mutant embryos appear to exhibit normal muscle development in the limbs. Consistent with this data, Braun et al. (1994) noted that embryos lacking \textit{Myf-5} exhibit normal migration of Pax-3-expressing myogenic precursor cells into the limbs. In addition, our data suggest that, while muscle development progressed apparently normally in the body proper of \textit{MyoD} mutant embryos, myogenesis in the limb buds was markedly delayed.

Delayed myogenesis in the limbs of \textit{MyoD}⁻/⁻ and not \textit{Myf-5}⁻/⁻ embryos

To confirm and extend this analysis, we employed immunohistochemistry with a panel of antibodies reactive with muscle proteins to further investigate skeletal myogenesis in the trunks and limbs of \textit{Myf-5} and \textit{MyoD} mutant embryos. We performed immunohistochemistry on transverse sections of embryos between 10.5 and 13.5 days post coitum (dpc) with antibodies reactive with proteins expressed in both myoblasts (desmin and nestin) and differentiated myotubes (myosin heavy chain and sarcomeric actin). Desmin and nestin are intermediate filament proteins expressed in both proliferating myoblasts and newly formed differentiating myocytes, whereas myosin heavy chain (MHC) and sarcomeric actin are expressed exclusively in terminally differentiated myotubes and myofibers (Bischoff, 1994; Hauschka, 1994). Qualitatively identical patterns of staining were observed with anti-desmin and anti-nestin antibodies as well as with anti-MHC and anti-actin antibodies. For clarity, we therefore only show immunohistochemistry with anti-desmin (Fig. 3) and anti-MHC (Fig. 4) antibodies.

Shown in Fig. 3 are sections of 12.5 and 13.5 dpc wild-type embryos stained with anti-desmin antibody D33 revealing the presence of myoblasts and differentiated myocytes in areas forming presumptive musculature (arrowheads) in the intercostal muscles of the trunk (Fig. 3A,G) and the muscle anlagen of the forelimbs (Fig. 3D,J). Note the decrease in intensity of desmin expression between 12.5 and 13.5 dpc. Companion sections stained with anti-MHC antibody MF20 are shown in Fig. 4. MHC is expressed following terminal differentiation; therefore, MF20 only stains differentiated myocytes (arrowheads) which were readily detectable in the intercostal muscles of the trunk (Fig. 4A,G) and the muscle anlagen of the forelimbs (Fig. 4D,J). Note the increase in distribution and intensity of MHC expression between 12.5 and 13.5 dpc.

Consistent with the observations of Braun et al. (1994), 12.5 dpc \textit{Myf-5} embryos displayed reduced numbers of desmin-expressing myocytes in the normal location of intercostal muscles in the trunk (compare Fig. 3A,C). However, the muscle anlagen of the limb buds in 12.5 dpc \textit{Myf-5}⁻/⁻ embryos contained a normal distribution of desmin-expressing myocytes (Fig. 3F). Although reduced in intensity, an analogous pattern of desmin expression was also observed in

---

**Fig. 2.** Delayed expression of the \textit{MD6.0-lacZ} transgene in the muscle anlagen of forelimbs in \textit{MyoD}⁻/⁻ embryos. At 10.5 dpc, no expression of \textit{lacZ} (arrows) was detectable in wild-type (A), \textit{MyoD}⁺/+ (B) or \textit{Myf-5}⁻/⁻ (C) embryos. By 11.5 dpc and through 12.5 dpc, \textit{lacZ} expression (arrows) revealed the onset of myogenic differentiation in wild-type (D,G), and \textit{Myf-5}⁻/⁻ (E,I) forelimbs, but examination of \textit{MyoD}⁻/⁻ embryos (E,H) revealed an absence \textit{lacZ} expression in the forelimbs (red arrowheads). By 13.5 dpc, development of limb musculature, as evidenced by \textit{lacZ} expression (arrows), was underway in wild-type (J), \textit{MyoD}⁻/⁻ (K) and \textit{Myf-5}⁻/⁻ (L) embryos.
the trunk and limbs of 13.5 dpc Myf-5−/− embryos (Fig. 3I,L). Similarly, 12.5 dpc Myf-5−/− embryos stained with anti-MHC antibody MF20 revealed a reduced distribution of MHC-expressing differentiated myocytes in the trunk (compare Fig. 4A,C) and a normal distribution in the limbs (compare Fig. 4D,F). By 13.5 dpc, Myf-5−/− embryos contained increased numbers of MHC-expressing myocytes in the trunk (Fig. 4I) and normal numbers in the limbs (Fig. 4L). Therefore, these data support our assertion that myogenic development occurs normally in the limbs of Myf-5 mutant embryos.

As suggested by the expression of MD6.0-lacZ, the muscle anlagen of the forelimbs of 12.5 dpc MyoD−/− embryos contained little or no detectable desmin-expressing myocytes (Fig. 3E). However, desmin-expressing myocytes were readily detectable in 12.5 dpc MyoD−/− embryos in the intercostal muscles of the trunk (Fig. 3B). By 13.5 dpc, desmin-expressing myocytes were detectable for the first time in the forelimbs of MyoD−/− embryos but at levels less than that in wild-type limbs (Fig. 3J). A normal distribution of desmin expression was observed in the trunk of 13.5 dpc MyoD−/− embryos (Fig. 3H). Examination of sections from 12.5 dpc MyoD−/− embryos stained with antibody MF20 revealed the presence of MHC-expressing myocytes in the trunk (Fig. 4B). However, no MHC-expression was detected in the limbs (Fig. 3E). Furthermore, whereas 13.5 dpc MyoD−/− embryos exhibited a normal distribution of MHC-expression in the trunk (Fig. 4H), only small numbers of MHC-expressing differentiated myocytes were observed in the limbs (Fig. 4K).

The expression pattern of MD6.0-lacZ, together with the immunohistochemical analyses described above, indicated that the muscle developmental program differs in significant ways between Myf-5- and MyoD-deficient embryos. In embryos lacking Myf-5, the development of musculature of the paraspinal and intercostal muscles was delayed, but the development of limb, brachial arch and neck musculature proceeded apparently normally. Conversely, in MyoD-deficient embryos, the development of trunk musculature appeared relatively normal, but was marked delayed in the limb buds,

Fig. 3. Detection of desmin-expressing myocytes in Myf-5 and MyoD mutant embryos. Wild-type embryos at 12.5 dpc contained desmin-expressing myocytes (indicated with arrowheads) in the intercostal muscles of the trunk (A) and the muscle anlagen of the forelimbs (D). MyoD+/− embryos also contained desmin-expressing myocytes in the intercostal muscles of the trunk (B). However, MyoD+/− embryos completely lacked desmin-expressing myocytes in the muscle anlagen of the forelimbs (E). Myf-5 embryos, displayed very few desmin-expressing myocytes in the lateral regions of the body proper (C), but contained normal numbers of desmin-expressing myocytes in the forelimb buds (F). By 13.5 dpc, desmin expression was somewhat decreased, however well formed muscles were observed in the trunk of wild-type (G), MyoD+/− (H) and Myf-5+/− (I) embryos. The distribution of desmin expression in the muscle anlagen of the limbs appeared similar between wild-type (J) and Myf-5−/− (L) embryos, and somewhat reduced in MyoD−/− embryos (K). Arrowheads indicate intercostal muscles in A,B,C,G,H and I. Arrowheads indicate muscle anlagen of the forelimbs in D,E,F,J,K and L. Black boxes indicate areas shown in panels from trunk and limbs of 12.5 dpc and 13.5 dpc embryos.
brachial arches and neck region with myogenesis not evident in these regions until 13.5 dpc. These data support the hypothesis that Myf-5 and MyoD are differentially required for the appropriate development of epaxial and hypaxial muscle respectively.

**Migration and development of myogenic progenitors cells in MyoD**

Development of trunk musculature has been previously reported to be delayed in embryos lacking Myf-5 (Braun et al., 1994). However, our results reveal that Myf-5 mutant embryos appear to exhibit normal muscle development in the limbs. Consistent with our data, Braun et al (1994) noted that embryos lacking Myf-5 exhibit normal migration of Pax-3-expressing myogenic-precursor cells into the limbs. Pax-3 is a member of the paired-box-containing transcription factor family and is expressed in a variety of neural and mesodermal cell types including the myogenic migratory cells that originate from the ventral-lateral region of the dermamyotome and migrate to the limb (Williams and Ordahl, 1994). To determine whether the migration of Pax-3-expressing cells from the dermamyotome to the limb bud occurred normally in the absence of MyoD, we performed whole-mount in situ hybridization with an antisense RNA probe generated from the mouse Pax-3 cDNA.

Examination of 36-somite (10.75) dpc embryos hybridized with a Pax-3 probe revealed a normal distribution of Pax-3-expressing cells migrating from the dermamyotome into the forelimbs and hindlimbs relative to wild type (compare Fig. 5A,B and C,D). Inspection of sectioned embryos similarly revealed a normal pattern of Pax-3-expressing cells extending from the lateral edge of the dermamyotome into the forelimb and hindlimb (not shown). Therefore, we conclude that the delayed onset of muscle differentiation in the limbs of MyoD**

---

**Fig. 4. Detection of myosin heavy chain (MHC)-expressing myocytes in Myf-5 and MyoD mutant embryos.** Wild-type embryos at 12.5 dpc contained detectable MHC-expressing myocytes (indicated with arrowheads) in the intercostal muscles of the trunk (A) and the muscle anlagen of the forelimbs (D). MyoD**

---

Myo-5 and MyoD are not expressed until after the Pax-3-expressing migratory cells have arrived in the limb bud (Sassoon et al., 1989; Tajbakhsh and Buckingham, 1994). One possible reason for the delay in limb muscle development was that induction of MyoD transcription in the limbs is required for the initiation of the muscle developmental program and subsequent
up-regulation of the other myogenic factors. To investigate the consequence of the absence of MyoD on expression of Myf-5 and myogenin, we performed immunohistochemistry with antibodies reactive with Myf-5, MyoD and myogenin proteins. Examination of MyoD expression in 11.5 dpc Myf-5-/- embryos revealed a reduced numbers of stained cells in myotome and trunk, and no detectable expression in the limb buds (not shown). However, consistent with the normal progression of muscle development in the limbs of Myf-5-/- embryos, we observed normal expression of MyoD protein in the nuclei of myogenic precursors in the forelimb buds of 12.5 dpc Myf-5 mutant embryos (compare Fig. 6A and C). The specificity of the antibody was confirmed by the observation that the anti-MyoD antibody did not stain myocytes in MyoD-/- embryos (Fig. 6B).

The observation that myogenesis was delayed in the limbs of MyoD-/- embryos suggested that we would observe a corresponding delay in the onset of Myf-5 expression. However, Myf-5 was expressed in a normal distribution and at seemingly elevated levels in the nuclei of myogenic precursors in the muscle anlagen of forelimb buds of 11.5 dpc (not shown) and 12.5 dpc embryos lacking MyoD (compare Fig. 6D and E). The specificity of the antibody was confirmed by the observation that Myf-5 protein was not detectable in Myf-5-deficient embryos (Fig. 6F). Consistent with this data, we have observed that expression of a knocked-in lacZ from the endogenous Myf-5 regulatory sequences (Tajbakhsh et al., 1996) was similarly unaffected by the absence of MyoD (unpublished observation). Therefore, in the developing MyoD mutant embryos, Pax-3-expressing myogenic precursors undergo normal migration into the limb and subsequently up-regulate Myf-5 in an apparently normal manner.

Immunohistochemical detection of myogenin protein revealed a markedly reduced distribution of stained nuclei in the muscle anlagen in MyoD-/- limbs (Fig. 7C,D) compared to wild-type (Fig. 7A,B) and Myf-5-/- limbs (Fig. 7E,F) at 12.5 dpc. An analogous delay in expression of a myogenin-lacZ transgene (Cheng et al., 1993) was also observed in the limbs of MyoD-/- embryos (not shown). These results therefore suggest that, in the limbs between 11.5 and 12.5 dpc, Myf-5

**Fig. 5.** Migration of Pax-3 myogenic precursors into the limbs occurs normally in MyoD-deficient embryos. Examination of whole-mount 36-somite (10.75 dpc) wild-type (A) and MyoD+/- (C) embryos hybridized with a Pax-3 probe revealed an identical pattern of distribution of Pax-3-expressing cells undergoing migration into the forelimbs (arrows), and in the dermamyotome (arrowheads). Detail of forelimb buds of wild type and MyoD+/- is shown in B and D, respectively.

**Fig. 6.** Myf-5 is expressed in the muscle anlagen of limb buds of MyoD mutant embryos. Immunohistochemistry with anti-MyoD antibody stained nuclei of myogenic precursors in the forelimb buds of 12.5 dpc wild-type (A) and Myf-5+/- embryos (C). MyoD protein was not detected in sections of MyoD+/- embryos (B). Immunohistochemistry with anti-Myf-5 antibody stained nuclei of myogenic precursors in the forelimb buds of 12.5 dpc wild-type (D) and in MyoD+/- embryos (E). Myf-5 protein was not detected in sectioned Myf-5+/- embryos (F). Arrowheads denote labeled cells within regions stained with anti-MyoD, or anti-Myf-5 antibody.
expression is insufficient to fully activate myogenin transcription resulting in about a 2 day delay in the muscle developmental program. Taken together with the observed reduced expression of other markers, these data suggest that, in the limbs of MyoD mutant embryos, myogenic precursor cells arrive and up-regulate Myf-5 normally, but display a markedly reduced ability to progress through their normal developmental program. Therefore, these findings support the hypothesis that Myf-5 cannot fully substitute for MyoD during the first 2 days of mouse limb bud development.

DISCUSSION

To further our understanding of the roles of Myf-5 and MyoD during embryogenesis, transgenic MD6.0-lacZ mice were bred with Myf-5 and MyoD mutant mice. In 10.5 to 12.5 dpc MyoD-/- embryos, MyoD-lacZ expression was virtually absent in the limb girdles and brachial arches, but was normal in the developing musculature of the trunk. By contrast, MyoD-lacZ expression in 11.5 to 12.5 dpc Myf-5-/- embryos was markedly reduced in the trunk, but was normal in the limb girdles and brachial arches. However, by 13.5 dpc, the first expression of the MyoD-lacZ transgene was detected in the limbs and brachial arches of MyoD-/- embryos. The distribution of desmin, nestin, sarcomeric-actin and myosin heavy chain proteins in sections from mutant embryos displayed similarly altered patterns of expression. Interestingly, we observed normal migration of Pax-3-expressing myogenic precursors into the limb and normal induction of Myf-5 expression in the absence of any expression of other muscle markers in the limbs of MyoD-deficient embryos.

MyoD mutant embryos exhibit about a 2 day delay in the development of hypaxial musculature. Myf-5 mutant embryos exhibit a similar delay in the development of epaxial musculature. The delay in the muscle development of the trunk was previously reported in Myf-5-deficient embryos (Braun et al., 1994). The authors observed that the onset of myogenesis was delayed until the induction of MyoD expression at about 13.5 dpc. However, our data clearly suggests that MyoD is sufficient for the normal activation and progression of the hypaxial myogenic program during development of Myf-5-/- embryos. Moreover, our data suggests that, Myf-5 is sufficient for the normal activation and progression of epaxial myogenesis in the absence of MyoD. However, although Myf-5 protein was up-regulated normally in 11.5 dpc limb buds of MyoD mutant embryos, myogenic development in the limbs was delayed until 13.5 dpc. Taken together, these observations strongly support the assertion that the Myf-5 and MyoD have unique functions in the development of epaxial (paraspinal and intercostal) versus hypaxial (limb and abdominal wall) musculature, respectively. Moreover, these data indicate that Myf-5 cannot fully substitute for MyoD in hypaxial muscle development.

Gene targeting and expression analysis has suggested the functional classification of the MRFs into two groups. Myf-5 and MyoD form the first group, which act as differentiation factors, and myogenin and MRF4 form the second group, which act as differentiation factors (Rudnicki and Jaenisch, 1995; Megeney and Rudnicki, 1995). Phylogenetic analysis and chromosomal location suggests that this subgrouping of the MRF family arose through evolution by successive gene duplication events (Atchley et al., 1994; Megeney and Rudnicki, 1995). According to this hypothesis, Myf-5 appears to represent the ancestral gene of the family, MRF4 arose from Myf-5 by gene duplication at the same locus, myogenin arose from MRF-4 after a gene duplication event to a second chromosome and, finally, MyoD arose from Myf-5 after a gene duplication event to a third chromosome. Our results clearly suggest important and unique roles for Myf-5 and MyoD in the development of epaxial and hypaxial musculature, respectively. It is interesting to speculate that the duplication of MyoD from Myf-5 may have allowed a subsequent evolutionary specialization.
of Myf-5 and MyoD in the development of epaxial and hypaxial musculature. Presumably, such functional specialization would have facilitated the development of jaw and limb musculature in the urochordate-like ancestors of the vertebrates.

Analysis of MRF expression during mouse somitogenesis suggests that the dermamyotome is divided into at least two subdomains as defined by two different MRF expression programs (Smith et al., 1994). For example, in forelimb somites, Myf-5 protein appears in the dorsal-anterior cells of the somites prior to formation of the dermamyotome on day 8 of gestation. MyoD protein first appears in a few cells in the ventral portion on day 9.5 and, by day 11.5, has spread throughout the dermamyotome with higher levels expressed in the ventral portion. Myf-5 and MyoD appear to be initially expressed in a mutually exclusive manner, but they are soon co-expressed in the majority of myogenic cells. (Smith et al., 1994; Cossu et al., 1996a). By contrast, Myf-5 and MyoD expression is concomitantly induced in the developing muscle anlagen in the limb bud (Sassoon et al., 1989; Tajbakhsh and Buckingham, 1994). Lastly, analysis of ES cells carrying targeted mutations in Myf-5 induced to differentiate in vitro into cultures containing skeletal myocytes has suggested that Myf-5 and MyoD are initially induced in distinct populations of myogenic stem cells (Braun and Arnold, 1996). Taken together, these data support the notion that the Myf-5 and MyoD have distinct roles in the ontogeny of epaxial and hypaxial musculature (Rudnicki and Jaenisch, 1995; Cossu et al., 1996b).

Differences in Myf-5 and MyoD activity may be defined at any of several levels and various possibilities can be hypothesized. Differences in the control of Myf-5 and MyoD expression may result in different levels of Myf-5 and MyoD protein in epaxial and hypaxial compartments. Myf-5 and MyoD may differentially interact with target promoters resulting in the activation of partially distinct sets of genes. Myf-5 and MyoD may be differentially regulated by signal transduction pathways, at the level of protein-protein interactions, or by phosphorylation. Lastly, the availability of E2A bHLH factors may differ between the trunk and the limb bud resulting in the formation of different sets of MRF-E2A heterodimers with different activities. Alternatively, small differences at all of these levels may contribute cumulatively towards defining unique activities of Myf-5 and MyoD. The introduction into the mouse germline of Myf-5 coding sequences into the MyoD gene, and conversely of MyoD sequences into the Myf-5 gene, should reveal whether Myf-5 and MyoD have intrinsic differences in activities, or alternatively, are differentially regulated at the level of transcription, during the development of epaxial and hypaxial musculature.

The Myf-5-expressing myogenic precursor cells in the limbs of 11.5 and 12.5 dpc MyoD appear to be present in a normal distribution (see Fig. 6), but are markedly delayed in their ability to progress through their normal developmental program for at least 2.5 days (Figs 1-4). This observation raises the question of the identity of these cells. One interesting possibility is that the Myf-5-expressing myogenic cells represent a stem cell population normally present in small numbers that is distinct from their MyoD- and desmin-expressing daughter cells. In adult skeletal muscle, the existence of such myogenic stem cells (also called satellite cells) that have an identity distinct from their desmin-expressing daughter cells has been suggested both by the biology of muscle regeneration (reviewed by Bischoff, 1994), and by studies of muscle regeneration in mice lacking MyoD (Megeney et al., 1996). However, whether putative myogenic stem cells actually exist during embryogenesis remains an open question. Clearly, further studies to establish the characteristics and properties of early myogenic cells during embryonic development should elucidate this issue.

The development of limb muscle differs in significant ways from dermamyotomal myogenesis in the trunk. For example, Myf-5 and MyoD are not expressed at detectable levels in the presumptive myogenic precursor cells migrating from the somite to the limb bud (Sassoon et al., 1989; Tajbakhsh and Buckingham, 1994). However, these migratory cells express Pax-3, a member of the paired-box-containing transcription factor family expressed in a variety of neural and mesodermal cell types (Williams and Ordahl, 1994). Homozygous Splotch mutant embryos lacking Pax-3, have normal trunk musculature, but are deficient in the formation of limb skeletal muscle (Bober et al., 1994; Goulding et al., 1994; Kothary et al., 1993). The Pax-3-expressing cells migrating from the somite to the limb bud are believed to be determined towards the muscle lineage because, in avian grafting experiments as well as in vitro, muscle differentiation is the observed result (Tajbakhsh and Buckingham, 1994; and references therein). However, it has been argued that this population is multipotential because they can contribute to non-muscle structures in vivo (Kiency et al., 1981). Recent experiments have indicated that Pax-3 plays an essential function leading to the activation of MyoD transcription during development (Maroto et al., 1997; Tajbakhsh et al., 1997). Here, we show that the migration of Pax-3-expressing myogenic precursors occurs normally into the limbs of MyoD-deficient embryos (Fig. 5). In addition, we have observed similarly normal migration of Pax-3-expressing cells into the limbs of embryos deficient for both Myf-5 and MyoD in the complete absence of any skeletal myogenesis (B. Kablar, and M. A. Rudnicki, unpublished observation). Taken together, our data therefore argue that the induction of Myf-5 and MyoD transcription in the muscle anlagen of the limbs occurs in a mutually autonomous manner and independently of axial myogenesis.

Our results strongly support the notion that the putative myogenic lineages that give rise to epaxial and hypaxial musculature have different requirements for Myf-5 or MyoD for appropriate development. Nevertheless, the presence of skeletal muscle in the limbs and trunks of newborn Myf-5- and MyoD-deficient animals suggests that eventually both cellular lineages can substitute for one another in the longer term resulting in an apparent functional redundancy by birth. However, deficiencies in particular muscle groups have been observed in the limbs of outbred MyoD mutant mice (P. Hallauer and K. Hastings, personal communication) and in the back of Myf-5 mutant mice (S. Tajbakhsh, personal communication). Clearly, a more detailed morphometric and histopathological analysis of trunk and limb musculature must be performed to elucidate the degree that Myf-5 and MyoD can functional substitute for one another in this regard.

We thank Eric Olson for providing the myogenin-lacZ mice and Linda May for expert technical assistance. M. A. R. is a Research Scientist of the National Cancer Institute of Canada, and a member of Myogenesis in Myf-5 and MyoD mutant mice 4737
REFERENCES


(Accepted 25 September 1997)