INTRODUCTION

The development of skeletal muscle during embryogenesis is a multistep process, which involves commitment of mesodermal progenitor cells to the myogenic lineage, enlargement of this cell compartment and differentiation events resulting in highly specialized postmitotic cells. Subsequently, these cells undergo maturation and diversification to form functional muscle tissue in response to various environmental and cell autonomous influences (reviewed by Cossu and Molinario, 1987).

A family of genes including MyoD, myogenin, Myf-5 and Myf-6 (MRF4) has been identified, which controls at least some of these developmental steps (reviewed by Weintraub et al., 1991; Arnold and Braun, 1993). Their role as key regulators of myogenic determination and differentiation events was proposed by tissue culture experiments in which all four factors can convert a variety of different cell types into muscle cells when expressed from constitutive promoters (reviewed by Olson and Klein, 1994).

Other important clues came from the developmental pattern of myogenic factor expression. In situ hybridization studies suggested a sequential activation of the factors in somites with Myf-5 being expressed first, followed by myogenin, Myf-6 and MyoD. Importantly, Myf-5 transcription precedes the appearance of contractile proteins suggesting that it may have a crucial role in the initiation of muscle development (reviewed by Buckingham, 1992; Arnold and Braun, 1993).

Mouse mutants, recently generated by homologous recombination in embryonic stem cells, have provided new tools to establish the role of myogenic factors in vivo and distinguish between individual functions of different family members. Inactivation of the MyoD gene has no severe effects upon skeletal muscle differentiation; however, we noted that Myf-5 expression remains high presumably compensating for the loss of MyoD (Rudnicki et al., 1992). Similarly, mice homozygous for a Myf-5 mutation do not display a striking muscle phenotype at birth, but die perinatally due to a severe malformation of the ribs (Braun et al., 1992). Inactivation of both the

SUMMARY

The expression pattern of myogenic regulatory factors and myotome-specific contractile proteins was studied during embryonic development of Myf-5 mutant mice by in situ hybridization and immunohistochemistry.

In contrast to somites in wild-type embryos, no expression of myogenin and Myf-6 (MRF4), or any other myotomal markers was detected in mutant animals at E9.0 and E10.0 indicating that Myf-5 plays a crucial role during this developmental period. Significantly, the onset of MyoD expression in rostral somites of E10.5 embryos was unaffected by the Myf-5 mutation suggesting that the activation of the MyoD gene occurs independently of Myf-5 at the correct developmental time. Immediately after the activation of MyoD myogenin transcripts and protein accumulated within the myotome. The first contractile proteins of the sarcomeric apparatus appeared slightly later. By E11.5 the expression of muscle markers were indistinguishable between wild-type and Myf-5 mutant mice.

The migration of muscle precursor cells that leave the somites to form limb musculature was monitored in Myf-5-mutant mice by Pax-3 expression. Pax-3-positive cells were equally found in somites and limbs of E10.0 wild-type and mutant mice indicating that myogenic factor expression at the level of somites is not a prerequisite for determination and subsequent migration of limb precursor cells.

Key words: MyoD, expression, skeletal myogenesis, Myf-5, mouse
**MyoD and Myf-5** genes, however, results in mice completely devoid of skeletal muscle lacking both myofibers and myoblasts (Rudnicki et al., 1993). In contrast to these results, targeted mutation of the **myogenin** gene leads to decreased myoblast differentiation resulting in muscles with only few myofibers but apparently normal numbers of nonmononucleated myoblasts (Hasty et al., 1993; Nabeshima et al., 1993). The mutant myoblasts express MyoD and are capable of differentiating into myotubes in culture (Nabeshima et al., 1993). These observations imply that Myf-5 and/or MyoD control the determination of myoblasts and that myogenin is involved in the terminal differentiation process.

We have shown previously that the skeletal musculature in **Myf-5** mutant mice appears essentially normal at birth, whereas no muscle-specific terminal differentiation markers were detected in somites of E10.5 mutant embryos (Braun et al., 1992). Based on these observations, we suggested that in the mutant myotome formation may be delayed but that eventually normal muscle cells form. In this paper, we present a detailed analysis of the expression of MyoD, myogenin and Myf-6 (MRF4), as well as sarcomeric marker genes, in **Myf-5**-mutant embryos in an effort to assess which of these genes may initiate muscle formation in somite development.

Our results show that in the absence of **Myf-5** myogenesis is considerably delayed and begins in cranial somites at E10.5 with the expression of MyoD. The activation of MyoD at the correct developmental time suggests that it is independent on **Myf-5**. Activation of MyoD therefore, constitutes a second distinct entry point into the myogenic program, in addition to and independent of **Myf-5** expression. We furthermore demonstrate that the migration of Pax-3-expressing cells from the dermomyotome to the limb buds is unaffected in **Myf-5** mutants, even in the complete absence of myogenic factors. These findings indicate that migratory dermomyotomal cells constituting the prospective muscle cell population of the limbs acquire their fate entirely independent of the myotome and any signals it may provide.

**MATERIALS AND METHODS**

**Mice**

The generation of **Myf-5** mutant mice has been described previously (Braun et al., 1992). Most of the analyses were performed with 129SvJMyf5m1 mice backcrossed two or three times to C57BL6 or BALB/c mice. Crosses of inbred 129SvJMyf5m1 mice generally yielded fewer embryos, which were slightly delayed in development but with virtually identical phenotype. Embryos were staged by the number of somites and by counting the appearance of the vaginal plug as day 0.5 p.c.

Material for genotyping of the embryos was obtained by dissection of yolk sac tissue from the embryo proper. Genomic DNA isolated from the yolk sac was cut with BamHI and analyzed on Southern blots using a probe that flanked the mutant **Myf-5** locus as described previously (Braun et al., 1992).

**In situ hybridization**

Sections of embryos were prepared for in situ hybridization and immunocytochemistry by fixation in 4% paraformaldehyde for 12 hours, followed by dehydration in 0.5 M sucrose in PBS overnight. Embryos were frozen in OCT (Miles Scientific) and sectioned on a cryostat.

Prehybridizations and hybridizations were performed with [35S]UTP-labelled cRNA probes as described (Ott et al., 1991; Sassoon et al., 1989). The cRNA probes for MyoD, myogenin, Myf-6 and Pax-3 were described by Sassoon et al. (1989), Bober et al. (1991) and Bober et al. (1994), respectively.

**Immunohistochemistry**

Immunocytochemistry was performed on cryostat sections using vectastain elite kits (Braun et al., 1989). The following antibodies were used: mAb MF20 raised against sarcomeric MyHC (kindly provided by D. Fishman, New York), mAb MY32 against fast MyHC (obtained from Sigma Inc.), monoclonal antibodies against desmin and titin (kindly provided by K. Weber, Göttingen), mAb F5D which reacts specifically with myogenin but not with other bHLH-proteins (kindly supplied by W. Wright, Dallas).

**RESULTS**

**Myogenic factors and markers for terminal muscle differentiation are absent in somites of E9.5 and E10.25 mutant Myf-5 embryos**

Our initial studies suggested that Myf-5 is required for the development of early somitic myocytes (Braun et al., 1992). To explore in more detail the **Myf-5** mutant phenotype in early to late somite stage embryos in situ hybridizations and immunocytochemical stainings were performed.

Previous work established that in wild-type mice **Myf-5** is expressed in the segmented paraxial mesoderm from E8 to E13 (Ott et al., 1991). Myogenin RNA first appears in the myotomes at E8.5 and continues to be expressed throughout fetal development (Sassoon et al., 1989). Myf-6 is expressed transiently at E10 and E11 and is re-expressed at E16. It constitutes the most abundant myogenic factor in postnatal life (Bober et al., 1991; Hinterberger et al., 1991). MyoD RNA is first detected at E10.5 and remains expressed throughout fetal development (Sassoon et al., 1989).

In contrast to wild-type E9.5 embryos (21-25 somites) in which myogenin transcripts were present on virtually every section, we were unable to detect myogenin mRNA in **Myf-5**-deficient mice of the same stage (Fig. 1A-D). To ascertain appropriate planes of sectioning through the dermomyotomal layer of somites, control hybridizations were performed on adjacent sections using a Pax-3 probe. Pax-3 is expressed in the dorsal part of the neural tube (Goulding et al., 1991), in the myotome and in the neighbouring dermatome (Bober et al., 1994). It constitutes an excellent marker for these compartments since all markers that are restricted to the myotome were negative in **Myf-5** mutant embryos at this stage and could therefore not be used to demonstrate the presence of dermomyotomal structures. As shown in Fig. 1L, a similar distribution of Pax-3 transcripts was found in somites of wild-type and mutant mice. This observation provides evidence that the amount of mesodermal cells within the dermomyotome is not greatly reduced in **Myf-5** mutants and that Pax-3 expression in the dorsal as well as in the ventral part of the dermomyotome is independent of **Myf-5**.

We next investigated the expression of MyoD in the same E9.5 wild-type and mutant embryos. As shown in Fig. 1E-H, no MyoD transcripts were found in wild-type or mutant embryos arguing against a compensatory or premature MyoD expression as a consequence of the **Myf-5** mutation. Weak
Fig. 1. Comparison of myogenin, MyoD and Pax-3 expression in genotyped wild-type (A,B,E,F,I,J) and Myf-5 mutant (C,D,G,H,K,L) embryos at the 22-somite stage. Adjacent transverse sections of trunk somites were hybridized with myogenin (B,D), MyoD (F,H) and Pax-3 (J,L) probes. Homozygous mutant embryos do not express myogenin mRNA at this stage, while Pax-3 mRNA is expressed in the dermomyotome at normal levels. No MyoD transcripts were observed in wild-type and mutant embryos. Corresponding bright-field illuminations are shown to the left of each dark-field panel. Magnifications are 320×.
MyoD in situ hybridization signals were first identified at the 35-somite stage (E10.25) in Myf-5 mutant embryos (Fig. 2A,B). Transcripts for myogenin and Myf-6 were still undetectable in these embryos, although they were readily identified in wild-type embryos of this stage (data not shown). In contrast, somitic expression of Pax-3 appeared normal (Fig. 2G-H) as already shown in 22-somite-stage (E9.5) embryos.

Several additional myogenic markers such as MyHC, titin, nebulin or desmin were also analyzed by in situ hybridization or immunocytochemistry and found to be absent from somites of E10.25 (32 somites) and E10.5 (40 somites) mutant embryos (Fig. 3D,F,G). This confirms that virtually no muscle cell differentiation occurred up to this developmental stage in the absence of Myf-5.

**Myogenesis in mutant Myf-5 embryos is initiated by MyoD which is expressed at the correct developmental time**

Because Myf-5 mutant pups had apparently normal muscle tissue at birth, it was possible that expression of other myogenic factors compensated for the lack of Myf-5 resulting in normal muscle development. MyoD seemed a good candidate for compensation since it is, albeit weakly, expressed in Myf-5 mutant embryos at the 35-somite stage when no other myogenic factors have yet been activated. We therefore closely followed the appearance of MyoD mRNA in 40-somite embryos (E10.75) and compared it to the expression of other myogenic differentiation markers. The rostrocaudal direction of somite development allows the simultaneous observation of mature and immature somites on appropriate sections of the same embryo and a direct assessment of the sequence of myogenic factor expression.

As shown in Fig. 4A,B, strong MyoD hybridization signals were detected in somites of Myf-5 mutant embryos at the 40-somite stage. The intensity and distribution of these signals were comparable to those in wild-type littermates (Fig. 4E,F). Hybridization of adjacent sections to a myogenin probe revealed that myogenin mRNA was also expressed in the mature cranial somites where MyoD was already present.

**Fig. 2.** No myogenic factor is expressed in Myf-5 mutant embryos (35 somites) until MyoD expression is initiated. In situ hybridization of cryostat sections of Myf-5 mutant embryos to MyoD (A,B), myogenin, (C,D), Myf-6 (E,F) and Pax-3 (G,H) probes. Hybridization of adjacent cranial sections demonstrate that MyoD is the first myogenic factor which is expressed in Myf-5 mutants. The first MyoD-positive somites are indicated by arrowheads. The strong Pax-3 hybridization signals ascertain the plane of the section through the dermomyotome. Left panel: dark-field illumination, right panel: bright field-illumination. Magnification is 250x.
However, in the less mature, caudal somites where MyoD expression was just beginning, no myogenin mRNA was found (Fig. 4C,D). Thus, the sequential appearance of myogenin and MyoD mRNA in wild-type embryos appeared to be reversed in the absence of Myf-5. This observation suggests that myogenin expression in mutant embryos is triggered by MyoD, whereas in wild-type embryos Myf-5 seems to activate myogenin transcription.

It has been shown previously that myogenin protein appears only 2 days after the accumulation of myogenin mRNA presumably due to translational control or decreased half life of the protein (Cusella-DeAngelis et al., 1992). Therefore, it was of interest to compare directly the appearance of MyoD RNA with that of myogenin protein and additional muscle-specific differentiation markers. For this, adjacent sections from cranial and caudal regions of 37-somite embryos were hybridized to a MyoD cRNA probe (Fig. 5A-C) and reacted with antibodies against myogenin (Fig. 5D-F) or MyHC (=Myosin Heavy Chain) (Fig. 5G-I), respectively. The first nuclei positive for myogenin protein appeared in cranial somites of Myf-5 mutant embryos when MyoD mRNA was already weakly expressed (Fig. 5A,D). In contrast, caudal somites of the same embryo which were still negative for MyoD also lacked myogenin protein (Fig. 5B,E). Similarly, other terminal differentiation markers, such as MyHC, were detected in somites after MyoD and myogenin activation (Fig. 5G-H). As a positive control, sections of wild-type embryos were reacted with anti-myogenin antibodies (Fig. 5F) and anti-MyHC antibodies (Fig. 5I). Both antibodies strongly stained myotomal cells of 37-somite wild-type embryos. These results suggest that myogenin protein synthesis follows immediately after the expression of MyoD and the accumulation of myogenin RNA in mutant Myf-5 embryos. Subsequently, other terminal differentiation markers follow.

Between E10.5 and E11.5 of embryo development the prospective muscle-forming areas increase from a small part in the somite to occupy a major portion of the mesodermal space (Kaufman, 1992). This rapid proliferation of myogenic cells appeared to be unaltered in Myf-5 mutant embryos. Fig. 6 compares the distribution of MyoD and myogenin RNA...
producing cells in limb sections of E11.5 embryos. No difference in intensity or frequency of myogenic factor producing cells could be detected between mutant (Fig. 6A-D) and wild-type (Fig. 6E-H) embryos. Similar results were also obtained using antibodies against MyHC, desmin and sarcomeric actin (data not shown). The development of deep axial muscles and superficial body wall muscles were also indistinguishable between wild-type and mutant embryos at E11.5.

**Limb muscle precursor cells migrate normally to limb buds in the absence of myogenic factors**

In recent studies, it has been demonstrated that the paired-box-containing gene Pax-3 is expressed in a continuous domain from the dermomyotome to the limb buds which is consistent with the idea that it marks cells migrating from the lateral edge of the dermomyotome (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). These cells most likely represent the prospective myoblasts of the limb (Ordahl and LeDouarin, 1992). We used Pax-3 as a molecular marker to investigate the fate of prospective limb muscle cells in E10.25 Myf-5 mutant mice (32 somites).

Interestingly, the migration of Pax-3-positive cells from the dermomyotome to the limb buds appeared to be unaltered in Myf-5 mutant (Fig. 7A) as compared to wild-type embryos (Fig. 7D). Pax-3-positive cells were found to leave the lateral edge of the dermomyotome and to populate the limb buds at a stage when no myogenic factor was present in somites or elsewhere in Myf-5 mutants. The absence of myogenin and Myf-6 was confirmed by in situ hybridization to adjacent sections of the same embryo (Fig. 7B,C). In contrast, wild-type embryos of this stage showed expression of both, myogenin and Myf-6 (Fig. 7E-F) in somites but not in limb buds. As already demonstrated in Figs 1 and 2, the dermomyotomal expression domain of Pax-3 was not reduced in mutant embryos at day 10.25. Furthermore, these cells were arranged in the prospective muscle-forming areas of the limb buds which is compatible with their fate as future limb muscle cells.

Taken together these observations suggest that myogenic factors and the formation of a myotome, as defined by expression of myotomal markers, are not necessary to initiate or maintain the migration of prospective muscle cells to the limb buds.

**Fig. 4.** MyoD precedes the expression of myogenin in the caudal somites of Myf-5 mutant embryos at the 40-somite stage. Adjacent sections of Myf-5 mutant (A-D) or wild-type (E-H) embryos were hybridized to MyoD (A,B,E,F) or myogenin (C,D,G,H) probes. In Myf-5 mutants myogenin transcripts were present only in MyoD-expressing somites, whereas in wild-type embryos myogenin mRNA is already present in the most caudal somites prior to MyoD mRNA. The most caudal MyoD-positive somite of the mutant embryo begins to accumulate myogenin transcripts (marked by the arrowhead). Left panel: dark-field illumination, right panel: bright-field illumination. Magnification is 250×.
DISCUSSION

The experiments described here were undertaken to analyze early myotome development and the expression pattern of myogenic bHLH genes in Myf-5 mutant embryos. In addition, we supply evidence that the migratory cells for the prospective limb muscles which arise in the dermomyotome develop independently of the expression of myogenic regulatory factors and of early myotome development.

Our observations demonstrate that myogenic differentiation can be initiated in two waves during somite development. The first wave is dependent on Myf-5 expression: lack of Myf-5 results in a loss of expression of the myogenic factors myogenin and Myf-6 and consequently in lack of myogenic differentiation. The second wave coincides with the activation of MyoD which occurs at the appropriate developmental stage independently of Myf-5 or any other myogenic bHLH factor.

In double-mutant mice that are defective for both Myf-5 and MyoD, no myocytes and presumably no myoblasts arise (Rudnicki et al., 1993). These observations nicely support a model for myogenesis, which appears strictly dependent on these two factors: if both waves of muscle development are prevented myogenic differentiation is completely abolished.

As skeletal muscle development appears to proceed normally when MyoD becomes activated even in the absence of Myf-5, MyoD gene activation marks an autonomous and alternative entry point for myogenic precursors into the muscle differentiation pathway.

The existence of two separate and apparently unlinked entries into skeletal muscle development, one controlled by Myf-5 and another controlled by MyoD, is an unexpected result. The lack of qualitative differences in fetal and perinatal skeletal muscle between wild-type and Myf-5 and MyoD mutant animals argues that Myf-5 and MyoD probably do not direct the formation of functionally different muscle lineages during prenatal development. It is rather likely that both genes receive and integrate different signals that ensure the establishment and propagation of one type of skeletal muscle progenitors. In this context, it is interesting to note that MyoD expression starts just before the massive growth of the muscle cell compartment occurs between E10.5 and E11.5, which might explain why muscle differentiation proceeds virtually normal at E11.5 in Myf-5 mutant mice.

Undoubtedly, Myf-5 and MyoD genes are activated in
response to different developmental signals; however, their biochemical functions in cells appears largely overlapping or redundant. This hypothesis is supported in MyoD knock-out mice, which lack a muscle phenotype most probably due to an enhanced expression of Myf-5 suggesting that Myf-5 can substitute for MyoD and may maintain late somitic muscle development. At present, it is not clear whether the lack of a muscle phenotype in MyoD knock-out mice is due to numerical increase of early Myf-5-dependent somitic myoblasts or whether Myf-5 is expressed de novo in mesodermal cells that were not yet committed to the muscle fate. This is not a trivial problem since, in the latter case, the Myf-5 promoter would have to be activated by the same signals that switch on the MyoD promoter during later somitic development. The other possibility would require an expansion of Myf-5-expressing early somitic cells in the absence of MyoD-positive myoblasts. In wild-type development MyoD-expressing cells largely replace the population of Myf-5-expressing myoblasts.

Interestingly, when homozygous mutant Myf-5 ES-cells were differentiated in vitro, skeletal muscle cells emerged at the same time as in wild-type ES-cells expressing the same set of muscle markers. Simultaneously, MyoD expression occurred earlier in Myf-5 mutant cells (Braun and Arnold, 1994). The premature expression of MyoD in vitro as compared to its unaltered expression in vivo in the Myf-5 mutant embryos may indicate that the MyoD gene is under negative regulatory influence in vivo repressing the occurrence of MyoD in early somitic myoblasts. This effect seems to be bypassed in vitro.

Given the view that Myf-5 and MyoD triggered waves of myogenic differentiation yield qualitatively similar muscle cells, it remains enigmatic whether these different entry points into the myogenic lineage serve distinct functions. If either of the pathways is dispensable for later myogenesis as long as the other is operative, why are there different pathways at all? An extreme view would be that the somitic myotome between E8.5 and E10.5 is an evolutionary remnant in todays animals unnecessary for muscle development but required for cell interactions within the somite. Such interactions might be essential to set up correct bound-

Fig. 6. The level of myogenin and MyoD expression is indistinguishable in E11.5 Myf-5 mutant and wild-type embryos. Limb sections of Myf-5 mutant (A-D) and wild-type (E-H) embryos were hybridized to myogenin (A,B,E,F) or MyoD (C,D,G,H) probes. Differences are no longer detectable suggesting that muscle cells development continues normal from E11.5. Left panel: dark-field illumination, right panel: bright-field illumination. Magnification is 320×.
recently (Smith et al., 1993; Hughes et al., 1993), although Myf-5 and MyoD-expressing myoblasts seem to be equivalent at early stages. The use of homozygous Myf-5 mutant ES-cells to generate chimeras and/or the employment of conditional Myf-5 mutations will allow a more detailed molecular and physiological analysis in the future.

In a recent study, Bober et al. (1994) have shown that the paired-box-containing gene Pax-3 is expressed in a population of migrating limb muscle precursor cells. In addition Pax-3 is expressed in dermomyotomal and myotomal regions which partially overlap the normal expression domains of Myf-5. In this study, we have demonstrated that Pax-3 displays an apparently normal expression pattern both in somites and in migratory limb muscle precursor cells of Myf-5 mutant embryos.

The apparently normal expression of Pax-3 in mesodermal

Fig. 7. Comparison of Pax-3, myogenin and Myf-6 expression in somites forelimbs of mutant Myf-5 (A-C) and wild-type (D-F) embryos at the 32-somite stage. Broken lines indicate the contours of the embryos. Adjacent cryostat sections were hybridized to Pax-3 (A,D), myogenin (B,E) and Myf-6 (C,F). Pax-3-positive cells are present in the forelimbs of Myf-5 mutants despite the absence of myogenin (B) and Myf-6 (C). Wild-type embryos show expression of myogenin (E) and Myf-6 (F) in addition to Pax-3 expression (D). Dark-field illumination only is shown. Magnification is 125x.
structures of the dermomyotome and the prospective limb muscle cells might indicate that the mesodermal cells that receive their myogenic commitment by the expression of Myf-5 are still present in mutant embryos. They probably maintain their mesodermal fate unless MyoD expression commences rescuing their myogenic determination. Later during development, however, undetermined mesodermal cells are most likely sequestered because double homozygous Myf-5 and MyoD mutant mice show no undifferentiated mesodermal tissue in positions where muscle is normally located (Rudnicki et al., 1993; Braun et al., unpublished observations). In addition, the normal migration of Pax-3-positive limb muscle precursor cells from dermomyotome of Myf-5 mutant embryos at a time when no myogenic factors are present demonstrates that migration of limb muscle precursors is independent from commitment involving myogenic bHLH proteins. These findings corroborate previous studies that were unable to locate myogenic bHLH gene expression in the most lateral dermomyotomal cells and in the population of migrating cells (Bober et al., 1991; Tajbakhsh and Buckingham, 1994). In addition, it seems now very unlikely that committed myogenic cells orchestrate the emigration of prospective limb muscle cells or have a pioneering influence on lateral dermomyotomal cells which have not received their final commitment.

In summary, we have demonstrated that Myf-5 is responsible for the first developmental events that are specific for the muscle cell lineage. A later wave of myogenic differentiation however, critically depends on expression of MyoD or alternatively on a sustained and enhanced expression of Myf-5.

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