Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse

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SUMMARY

The limb muscles of vertebrates are derived from precursor cells that migrate from the lateral edge of the dermomyotome into the limb bud. Previous studies have shown that the paired domain-containing transcription factor Pax-3 is expressed in the limb in cells that are precursors for limb muscles (Williams, B. and Ordahl, C. P. (1994) Development 120, 785-796). In splotch (Pax-3−) embryos, the limb muscles fail to develop and cells expressing Pax-3 are no longer found in the limb. In this paper we have analyzed the role of Pax-3 in the migration and subsequent differentiation of limb muscle precursors. By labeling somites adjacent to the prospective forelimb with the lipophilic dye Dil, we have shown that cells derived from these somites do not migrate into the limbs of splotch mice. The failure of limb muscle precursors to invade the limb in splotch mice is associated with the absence of c-met expression in premigratory cells, together with a change in the morphology of the ventral dermomyotome. In addition, we have shown the lateral half of somites derived from day E9.25 splotch embryos can undergo muscle differentiation when grafted into the limb bud of stage 20 chick host embryos. Our results indicate that Pax-3 regulates the migration of limb muscle precursors into the limb and is not required for cells in the lateral somite to differentiate into muscle.

Key words: Pax-3, c-met, limb muscle migration, somite morphogenesis

INTRODUCTION

In vertebrate embryos the skeletal muscles are derived from two bilateral rows of somites located on each side of the neural tube. Newly formed somites consist of an epithelial ball of cells that differentiates into sclerotome and dermomyotome under the influence of signals from the ventral midline (Dietrich et al., 1993; Pourquie et al., 1993; Goulding et al., 1994; Koseki et al., 1993; Fan and Tessier-Lavigne, 1994). The dermomyotome in turn gives rise to dermis and to muscle. Skeletal myoblasts are generated from two populations of cells in the dermomyotome. The axial muscles are derived from the medial part of the somite and limb muscles develop from the lateral somite (Ordahl and Le Douarin, 1992). The appearance of the axial myotome beneath the dorsomedial lip of the dermomyotome is the first morphological evidence of skeletal muscle differentiation, with these cells giving rise to the axial musculature.

The appendicular muscles and body wall muscles are derived from the lateral half of the somite that is lacking a myotome (Ordahl and Le Douarin, 1992). Studies in chick and rat embryos demonstrate that the precursors of the limb musculature are derived from the lateral portion of the dermomyotomes that are immediately adjacent to the limb buds (Chevallier et al., 1977; Christ et al., 1977; Lee and Sze, 1993; Williams and Ordahl, 1994). These cells emigrate from the dermomyotome into the limb bud where they differentiate and fuse to form the primary myotubes of limb muscles. Both the lateral and medial somite halves are capable of generating either limb or trunk muscles in the three most caudal somites (Aoyama and Asamoto, 1988; Ordahl and Le Douarin, 1992) and this bipotentiality is lost as the somites mature further. This restriction of cell fate is due to the patterning of the somites by signals from the notochord, neural tube and adjacent tissues such as the lateral plate (Kenny-Mobbs and Thorogood, 1987; Rong et al., 1992; Brand-Saberi et al., 1993; Dietrich et al., 1993; Pourquie et al., 1993, 1995; Goulding et al., 1994).

A number of genes are expressed in early somites and in muscle precursor cells where they control myogenic differentiation. The helix-loop-helix (HLH) transcription factors MyoD, Myf-5, myogenin and Myf-6 (MRF4) are able to promote myogenesis in 10T1/2 fibroblasts (Olson, 1990). The expression of myf-5, myogenin and MyoD marks the emerging axial myotome in the embryo. In the mouse, myf-5 is expressed first in the dorsomedial lip of the dermomyotome (Ott et al., 1991), followed by the expression of myogenin and myoD in the myotome (Sassoon et al., 1989; Yee and Rigby, 1993), whereas in avian embryos myoD is expressed first (Pownall and Emerson, 1992). The role of all four myogenic regulatory factors in the development of skeletal muscle has been
analyzed in mice by gene targeting. These studies have shown that the presence of either MyoD or myf-5 genes in mouse embryos is sufficient for the survival and early proliferation of myoblasts (Braun et al., 1992; Rudnicki et al., 1992). The loss of myf-5 or MyoD function alone appears to have little or no effect on the development of skeletal muscle, while the loss of both genes results in the complete absence of myoblasts in embryonic day (E)11 embryos (Rudnicki et al., 1993). Skeletal myogenesis is severely disrupted in mice lacking an intact myogenin gene. In these mice the limb and body wall muscles fail to develop and the axial musculature is severely disorganized (Hasty et al., 1993; Nabeshima et al., 1993). In myf-6 (MRF-4) null mice skeletal myogenesis is largely unaffected, with some reduction in the size of the axial muscles of the back (Braun and Arnold, 1995). The analysis of the myf-6 null mice is complicated by the associated down-regulation of myf-5 in these mice.

Previous studies have shown that a number of paired box (Pax) genes that are expressed during somitogenesis may play a role in the differentiation of somites (Jostes et al., 1990; Goulding et al., 1991; Brand-Saberi et al., 1993; Bober et al., 1994; Goulding et al., 1994). Two Pax genes, Pax-3 and Pax-7, are expressed in the segmental plate and subsequently become restricted to the dermomyotome in response to signals from the notochord and floor plate (Goulding et al., 1994). In newly formed somites Pax-3 and Pax-7 are expressed throughout the dermomyotome. This expression pattern is modified as the somites differentiate further, such that Pax-3 expression is up-regulated in the ventrolateral dermomyotome, while the expression of Pax-7 is down-regulated (Goulding et al., 1994; Pourquie et al., 1995). Recently it has been shown that expression of Pax-3 in the ventrolateral dermomyotome is regulated by signals derived from the lateral plate and intermediate mesoderm (Pourquie et al., 1995). In older stage 21-25 chick embryos the expression domains of Pax-3 and Pax-7 overlap with the axial myotome (Goulding et al., 1994). Pax-3 transcripts are also found in the limb, in cells that are the precursors for the limb musculature (Williams and Ordahl, 1994). In contrast to Pax-3, Pax-7 is not expressed in the developing limb (Jostes et al., 1990; M. Goulding, unpublished results).

The pattern of Pax-3 expression in mouse embryos appears to be identical to that observed in the chick. In E10 embryos, cells in the mouse forelimb bud expressing Pax-3 appear to be contiguous with the domain of Pax-3-expressing cells in the dermomyotome (Bober et al., 1994), consistent with the proposal that these Pax-3-expressing cells are derived from the adjacent somites. While the somitic origin of Pax-3-expressing cells in the limb has been elegantly demonstrated in avian embryos by transplanting quail somites next to the developing wing bud

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**Fig. 1.** Expression of desmin and sarcomeric myosin in limb bud cultures from E11 wild-type and splotch embryos. E11 limb buds were dissociated and cultured for 4 days in vitro. Expression of desmin and sarcomeric myosin was analyzed by immunohistochemistry using a HRP-conjugated secondary antibody. (A,B) Desmin expression in 4-day limb cultures from wild-type (A) and splotch (B) embryos. The arrow marks a desmin-positive cell in A. (C,D) MF20 staining of 4-day limb cultures from wild-type (C) and splotch (D) embryos. The arrow in C marks a cell expressing sarcomeric myosin.

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**Fig. 2.** Cell migration from the somite into the limbs of wild-type and splotch embryos. Dil was injected into somites between E9.25 and E9.5 at the forelimb level. Embryos were fixed and 75-mm vibratome sections were collected from the forelimb region of wild-type (A,B) and splotch (C,D) embryos. The arrows in A and B mark Dil-labeled cells that have migrated into the limbs of E10.5 wild-type embryos. Note the elongated morphology of the cells in B. The arrowheads in D mark two Dil-labeled cells that are close to the site of injection in E10.5 splotch embryos. fl; forelimb, s; somite; v, ventral body wall.
Limb muscle precursors in splotch mice (Williams and Ordahl, 1994), the somitic origin of Pax-3-expressing cells in the mouse limb can only be inferred from studies in the chick and rat.

Mutations to the Pax-3 gene are responsible for the phenotype of the mouse mutant, splotch (Epstein et al., 1991, 1993; Goulding et al., 1993b; Vogan et al., 1993). A variety of mutations have been identified in the Pax-3 gene in five splotch alleles. In Sp r and Sp 4H the Pax-3 gene is deleted in its entirety (Epstein et al., 1991; Goulding et al., 1993b), while Sp 2H mice harbor a 32-base pair deletion that truncates the Pax-3 protein at Ala 237, deleting the highly conserved homeodomain and a C-terminal activation domain (Epstein et al., 1991). In the original splotch allele, Sp, the splice acceptor site for exon 4 is mutated and as a result produces aberrant Pax-3 transcripts either with a premature stop signal or lacking the 45 amino acids encoded by exon 4 (Epstein et al., 1993; Goulding et al., 1993b). The hypomorphic splotch allele, Sp d, contains a point mutation in the highly conserved N terminus of the paired domain (Vogan et al., 1993). Homozygous splotch embryos exhibit a variety of developmental defects that affect many Pax-3-expressing tissues in the embryo. Both heterozygous and homozygous splotch embryos have defects in neural crest-derived tissues that may result from changes in the migration of neural crest cells from the neural tube (Moase and Trasler, 1989), while homozygous splotch embryos develop spina bifida and, to varying degrees, exencephaly (Auerbach, 1954).

Fig. 3. Expression of c-met in the ventral dermomyotome of wild-type and splotch embryos. (A,B) Expression of c-met in the forelimb of E9.25 (A) and E9.5 mouse embryos (B). Note the c-met-expressing muscle precursor cells in the ventral dermomyotome and in the proximal region of the forelimb (see arrows). (C,D) Whole-mount in situ showing c-met expression in a E9.25 wild-type (C) and E9.25 splotch embryos. Note the lack of any c-met transcripts in the ventral dermomyotomes of the splotch embryo (cf. arrows in C and D). dm, dermomyotome; fl, forelimb; sc, spinal cord.

Fig. 4. Morphology of somites in wild-type and splotch embryos. Thin sections were collected through the forelimb of wild-type (A,C) and splotch (B,D) embryos at day E9.5, stained with toluidine blue or neutral red and examined by light microscopy. The arrows in A and B mark the ventral dermomyotome. The open arrows mark the axial myotome. The narrow arrow in C indicates cells with elongated processes that are still attached to the basal surface of the dermomyotome in wild-type embryos. The bold arrow marks the axial myotome. The narrow arrow in D marks cells in the ventral dermomyotome with a mesenchymal morphology in splotch embryos. The bold arrow marks the ventral lip of the dermomyotome and the open arrow the ventral edge of the axial myotome. dm, dermomyotome; fl, forelimb.
Previous studies have identified a defect in limb muscle development in embryos homozygous for the splotch mutation (Franz, 1993; Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994). This phenotype is characterized by the loss of Pax-3-expressing cells in the limbs of homozygous E10 splotch embryos and by the absence of limb myoblasts in older (E12-13) splotch embryos, as determined by the loss of myf-5, myoD and myogenin expression (Bober et al., 1994) and muscle-specific acetylcholinesterase activity (Goulding et al., 1994). Interestingly this loss of limb muscles occurs in all four alleles of splotch that have been characterized, including the hypomorphic Sp2 allele (Franz, 1993), while the axial muscles and ventral body wall muscles appear to be largely unaffected in all splotch alleles. The exact role Pax-3 plays in the generation of limb muscles is not clear. Pax-3 may control the migration of limb muscle precursors into the early limb bud and/or the later myogenic differentiation of Pax-3-positive muscle precursors in the limb. While the absence of Pax-3-expressing cells in the limb bud of splotch mice is consistent with a defect in cell migration, this could also be explained by the inappropriate down-regulation of Pax-3 expression in migrating muscle precursors in splotch embryos. In order to clarify the exact role Pax-3 plays in limb muscle development, we have undertaken a detailed investigation into the nature of the limb muscle defect in splotch mice. We have analyzed (1) the migration of presumptive limb muscle precursors in splotch mice, and (2) the ability of cells derived from somites to differentiate into muscle when transplanted into the limb. Our results show that Pax-3 is required for the correct morphogenesis of the dermomyotome and for the subsequent migration of limb muscle precursors from the dermomyotome into the limb bud. In addition we have used somite transplantation experiments to show that cells in the lateral half of newly formed somites from splotch embryos can differentiate into myoblasts when transplanted into a chick host limb.

MATERIALS AND METHODS

Mouse and chick embryos

The mice used in this study were originally obtained from the Jackson Laboratory, Bar Harbor, Maine and from the MRC Radiobiology Laboratory, Harwell, UK. Wild-type and mutant mouse embryos were obtained from timed matings of C57BL/6, Sp heterozygous and Sp2H heterozygous mice. The morning of the appearance of the plug was designated day 0.5. Eggs from fertile White Leghorn hens were obtained from MacIntyre Poultry Farms, San Diego, CA. Homozygous splotch embryos were identified by the presence of spina bifida and exencephaly from E9 onwards. The genotypes of embryos were confirmed by PCR using primers specific for the Sp allele (Franz, 1993), while the axial muscles and ventral body wall muscles appear to be largely unaffected in all splotch alleles. The exact role Pax-3 plays in the generation of limb muscles is not clear. Pax-3 may control the migration of limb muscle precursors into the early limb bud and/or the later myogenic differentiation of Pax-3-positive muscle precursors in the limb. While the absence of Pax-3-expressing cells in the limb bud of splotch mice is consistent with a defect in cell migration, this could also be explained by the inappropriate down-regulation of Pax-3 expression in migrating muscle precursors in splotch embryos. In order to clarify the exact role Pax-3 plays in limb muscle development, we have undertaken a detailed investigation into the nature of the limb muscle defect in splotch mice. We have analyzed (1) the migration of presumptive limb muscle precursors in splotch mice, and (2) the ability of cells derived from somites to differentiate into muscle when transplanted into the limb. Our results show that Pax-3 is required for the correct morphogenesis of the dermomyotome and for the subsequent migration of limb muscle precursors from the dermomyotome into the limb bud. In addition we have used somite transplantation experiments to show that cells in the lateral half of newly formed somites from splotch embryos can differentiate into myoblasts when transplanted into a chick host limb.

Embryos were cultured in vitro according to the method of Cusella-De Angelis et al. (1994). After 4 days, cultures were washed with PBS and fixed for 5 minutes in 4% paraformaldehyde prior to antibody staining. Fixed cells were washed extensively with PBS and stained with monoclonal antibodies to desmin (D3, Developmental Hybridoma Studies Bank) and sarcomeric myosin (MF20, Developmental Hybridoma Studies Bank) using standard procedures.

Dil injection and mouse embryo culture

Heterozygous Sp and Sp2H mice were mated to generate homozygous splotch (Pax-3-) embryos. E9.25 embryos were isolated from their decidua by careful dissection (Hogan et al., 1986) and those with an intact visceral yolk sac were placed in 5 ml of 80% rat serum and 20% Tyrodé’s buffer in 20 ml roller culture bottles. Embryos were cultured at 37°C for 4 hours in New Cultures prior to Dil injection (New et al., 1976). Control (wild type and heterozygous, n=32) and mutant (n=11) embryos were injected using a pressure-driven picopipetizer. A small bolus of Dil (approx. 15-20 nl; 5 mg/ml in dimethylformamide) was injected unilaterally in the lateral halves of somites 7-9 that lie adjacent to the developing forelimb. Embryos were then cultured in vitro for a further 24-30 hours to allow further development, before being dissected out and fixed in 4% paraformaldehyde. Dil-labeled cells were visualized under rhodamine epifluorescence using a Nikon Optiphot microscope, in either whole-mounted embryos or in vibratome sections (75 μm) through the forelimb.

Isolation and transplantation of mouse segmental plate and somites

E9.25 mouse embryos were removed from their decidua and the caudal region containing the last three somites and segmental plate was isolated by dissection using sharpened tungsten knives and placed in calcium- and magnesium-free phosphate-buffered saline containing 0.5% trypsin and 0.25% pancreatin for 10-15 minutes on ice (Sturm and Tam, 1993). In E9.25 embryos these somites are located at the level of the hindlimb. Tungsten knives were then used to dissect away any adhering tissues including the neural tube and surface ectoderm, and somites were divided into lateral and medial halves. A small portion of the lateral plate mesoderm was left attached to the somites to help orient the lateral and medial somite halves. This was then removed prior to implantation into the chick hosts.

Stage-20 chick embryos were used as hosts for the somite transplantation experiments. Eggs from White Leghorn hens were incubated for 3 days at 37.6°C in a humidified forced-draft incubator until they reached stage 20, according to Hamburger and Hamilton (1951). The embryonic membranes were cut and folded back over the right forelimb and an incision was made in the right forelimb just above the apical ectodermal ridge. The lateral half of either somites I-III or an equivalent length of segmental plate from E9.25 embryos was carefully inserted into the host chick limb by gently prying open the incision and maneuvering the graft tissue into the centre of the limb bud. Following the operation, eggs were sealed and returned to the incubator for a further 36-48 hours to allow further development of the limb.

In situ hybridization

Embryos were removed and immediately fixed in 3.5% formaldehyde in 50 mM phosphate buffer, pH 7.2. Following fixation, embryos were processed for cryostat sections (Goulding et al., 1993a). Transverse sections (10 μm) through the forelimb region of operated embryos were collected on gelatin-subbed slides and pre-treated for in situ hybridization. A 0.9-kb fragment encompassing the coding region of the mouse myogenin gene was used to generate 35S-labeled riboprobe specific for the mouse myogenin gene. In situ hybridization was performed as previously described (Goulding et al., 1993a). Sections were counterstained with Giemsa and photographed using a Nikon Optiphot microscope with dark field/bright field illumination. Whole-mount in situ hybridization was performed as described in
Goulding et al. (1993). The following probes were used for whole-mount in situ hybridization: Pax-3, 560-bp HindIII/PstI fragment (Goulding et al., 1991); Pax-7, 914-bp EcoRI fragment (Jostes et al., 1990); Sim-1, 450-bp fragment (Fun and Tessier-Lavigne, 1994). A 787-bp c-met cDNA probe containing sequences that encode part of the extracellular domain was generated by PCR.

Detection of myogenin expression in limb transplants using RT-PCR

Forelimbs from stage-25 chick host embryos were dissected free of adjoining tissues using sharpened tungsten knives. In each experiment, somites were transplanted into the right forelimb, while the unoperated left forelimb was used as a control. Total RNA was prepared from matched pairs of single isolated forelimbs by homogenizing them in guanidinium thiocyanate followed by phenol:chloroform extraction (Chirgwin et al., 1979). cDNA was synthesized from 5 μg of total RNA by reverse transcription using random hexamer oligonucleotides (Boehringer Mannheim). PCR amplification of mouse myogenin transcripts (35 cycles) was performed using oligonucleotides specific for the mouse myogenin gene that spans an intron. Control PCR reactions (25 cycles) were performed using oligonucleotide primers specific for either the mouse or chicken GAPDH gene:

myogenin 5’ primer: CTACCTTCTTGGTCACCTTCAC; myogenin 3’ primer: CGCCTCTGTAGCGGAGATCG;
Universal 5’ GAPDH primer: AAGGTCATCCCAGAGCTGAA;
Mouse 3’ GAPDH primer: GCCATGAGTTCACCCACCTC;
Chick 3’ GAPDH primer: ACCATCAAGTCCACAACAG.

RESULTS

splotch limbs do not generate myogenic cells in culture

Previous studies have demonstrated that limb muscles fail to differentiate in splotch embryos (Franz, 1993; Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994). We examined the limbs derived from E11 splotch and wild-type embryos for the ability to generate myoblasts in cultures of dissociated limb tissues. After 4 days in culture, the expression of two early myogenic markers, desmin and sarcomeric myosin (MF20), was examined by antibody staining (Fig. 1). In wild-type limb explants, scattered cells expressing desmin and sarcomeric myosin were observed throughout the cultures (Fig. 1A,C), while in splotch limb explants no desmin or sarcomeric myosin-positive cells were detected (Fig. 1B,D). These results provide further evidence that the limbs of splotch embryos lack a population of precursors capable of differentiating into muscle.

Limb muscle precursors fail to migrate in splotch embryos

In order to analyze the migration of the presumptive limb muscle precursor cells in wild-type, splotch heterozygote and splotch homozygote embryos, DiI was injected unilaterally into the lateral half of three somites located immediately adjacent to the developing forelimb bud between E9.25 and E9.5. Embryos were transferred to roller bottle cultures and allowed to develop in vitro for a further 24-30 hours, thus enabling the presumptive muscle precursors to migrate from the somites into the limb. Our analysis was restricted to 30 hours development in culture, as no further growth or development of embryos was observed after 30 hours. In addition, culturing embryos for longer than 30 hours resulted in a significant increase in the number of dead or dying embryos.

Examination of DiI-labeled cells in wild-type (n=8) and in heterozygous (n=12) embryos revealed extensive colonization of the limb buds by cells derived from somites injected with DiI (Fig. 2A,B). In the proximal region of the forelimb, two streams of DiI-labeled cells were observed (Fig. 2A). These two streams show a similar distribution to that of Pax-3-expressing cells present in the mouse forelimb between day E9.5 and E11 (Fig. 2C, Bober et al., 1994; Goulding et al., 1994), and appear to be analogous to the Pax-3-positive cells in the avian limb that have been previously identified as migrating muscle precursors (Williams and Or Dahl, 1994).

In control (wild-type and heterozygous) embryos, labeled cells were located as far as 300 μm from the site of injection. Careful examination of the morphology of DiI-labeled cells in wild-type embryos showed that a number of DiI-labeled cells exhibited an elongated morphology with cell processes oriented parallel to the direction of cell migration (see arrows, Fig. 2B). The elongated morphology of these cells supports our hypothesis that these DiI-labeled cells present in the forelimb are in the process of migrating from the dermomyotome into the limb. In the occasional injected wild-type or heterozygous embryo, only a small number of DiI-labeled cells were found in the limb (n=3/20 embryos). This lack of colonization of the limb by DiI-labeled cells probably reflects differences in the timing and/or placement of DiI injections in these embryos such that the premigratory population of limb muscle precursors was poorly labeled.

In contrast to the extensive colonization seen in control embryos (wild-type and heterozygotes), no DiI-labeled cells were detected in the limbs of embryos homozygous for the splotch mutation (n=7; Sp/Sp, n=4; Sp<sup>2H</sup>/Sp<sup>2H</sup>, n=3). Occasionally, a few DiI-labeled cells were observed close to the injection site. These DiI-labeled cells numbered fewer than 2-3 cells per section and in all cases were located in the proximal part of the limb bud, close to the injection site (see arrowheads in Fig. 2D). In none of the injected splotch limbs were DiI-labeled cells observed further than 30-40 μm from the site of injection, whereas in wild-type embryos labeled cells were located at distances up to 300 μm from the injection site. The absence of DiI-labeled cells in the limbs of splotch embryos, together with the loss of Pax-3-expressing cells, is strong evidence that the presumptive limb muscle precursors do not migrate in splotch embryos.

c-met is absent from the ventral dermomyotome of splotch embryos

Recent studies analyzing the function of the c-met receptor protein tyrosine kinase have demonstrated a necessary role for c-met in the migration of limb muscle precursors in the mouse (Bladt et al., 1995). In E9-9.5 wild-type embryos, c-met is expressed in the ventral dermomyotome immediately adjacent to the forelimb (Fig. 3A-C). In E10 embryos, c-met is expressed in the ventral dermomyotomes at the level of the hindlimb, reflecting the temporal delay in the migration of hindlimb muscle precursors with respect to forelimb muscle precursors that occurs in the mouse (data not shown). In E9.25 embryos, expression of c-met at the forelimb level appears to segregate with cells that are beginning to invade the limb bud (Fig. 3B), consistent with previous studies indicating c-met is
expressed in limb muscle precursors (Sonnenberg et al., 1993; Bladt et al., 1995). In contrast to wild-type or heterozygote embryos, c-met expression was completely absent from the ventral dermomyotomes of E9.25 homozygous splotch embryos (Fig. 3D, cf. 3C). The loss of c-met expression in limb muscle precursors, together with the results of the Dil injection analysis, provide strong evidence that limb muscle precursors are unable to migrate into the limbs of splotch embryos.

### The structure of the dermomyotome is altered in splotch embryos

To further characterize the nature of the limb muscle defect we examined the morphology of somites immediately adjacent to the limb buds of wild-type and splotch embryos. Semi-thin (2-3 μm) plastic sections through the forelimb region were collected and stained with either toluidine blue or neutral red. In sections through E9.5 wild-type embryos, the dermomyotome consists of an epithelial structure that extends from the dorsal edge of the neural tube ventrally past the dorsal margin of the developing limb bud (Fig. 4A). In wild-type embryos the axial myotome can be seen beneath the dorsomedial two thirds of the dermomyotome and is absent from the ventrolateral region of the dermomyotome. In wild-type E9.5 embryos, we were also able to observe cells with an elongated morphology at the ventral edge of the dermomyotome in somites adjacent to the forelimb (Fig. 4C). These cells possessed elongated cell processes that were oriented toward the developing limb, suggesting they were in the process of migrating into the limb bud. Homozygous splotch embryos exhibited significant differences in the structure of the dermomyotome. The most noticeable feature of these somites was the absence of any epithelial structure where the ventral portion of the dermomyotome is normally located (Fig. 4B,D). Instead, the dermomyotome ends at the same level as the ventral lip of the axial myotome. These differences in morphology were apparent in E9.25 embryos but not in E8.5 embryos (data not shown), suggesting that the ventral dermomyotome is initially intact and that cells in the ventral dermomyotome subsequently lose their epithelial organization at a time when limb muscle precursors begin to migrate from the somite into the limb.

In previous studies of Pax-3 expression in chick somites, we have shown that Pax-3 and Pax-7 are initially expressed...
uniformly throughout the dorsolateral half of newly formed somites in the presumptive dermomyotome (Goulding et al., 1994).

During maturation of the somite, Pax-3 expression is upregulated in the ventrolateral third of the dermomyotome, whereas Pax-7 is down-regulated in these cells. This raised the possibility that changes to the epithelial structure of the dermomyotome in splotch embryos might be related to dynamic changes in the expression of Pax-3 and Pax-7 that occur as the somite matures. When Pax-3 expression was analyzed in E9.5 embryos by whole-mount in situ hybridization, a striking difference in the expression pattern was observed between wild-type and splotch embryos. In E9.5 wild-type embryos, Pax-3 expression marks the outline of the dermomyotome and extends ventrally past the dorsal margin of the forelimb bud (Fig. 5A, see arrow). In E9.5 splotch embryos, the expression of Pax-3 in the dermomyotome does not extend ventrally to the dorsal edge of the limb bud, and as a result the dermomyotomes appear truncated (Fig. 5B). The loss of this ventrolateral domain of Pax-3 expression can be seen in transverse sections through the forelimb region (compare Fig. 5H and I).

Close examination of sections through the forelimb region indicate that Pax-3 is down-regulated in those dermomyotomal cells that have lost their epithelial morphology. Prior to day E9.0, Pax-7 is expressed throughout the dermomyotome in a pattern that is indistinguishable between wild-type and splotch embryos. By E9.5 the expression pattern of Pax-7 in splotch somites is more disorganized than in wild-type or heterozygote embryos (Fig. 5D). In wild-type E9.5 embryos, Pax-7 expression is seen in the medial part of the dermomyotome but not in the ventral dermomyotome (Fig. 5G). In splotch embryos expression of Pax-7 is also retained in the medial dermomyotome but not in the ventral dermomyotome. Thus the change in cell morphology in the ventral portion of the dermomyotome appears to be closely associated with the downregulation of Pax-7 expression in these cells.

To further investigate the nature of these changes in the dermomyotome of splotch embryos, we examined the expression of Sim-1 within the dermomyotome. Sim-1 is a homologue of the Drosophila single-minded gene and is expressed strongly in the ventral third of the dermomyotome between E9.5 and E10.5 (Fan et al., 1996). We have used this marker to further
analyze the fate of cells in the dermomyotome of splotch embryos. In wild-type E9.5 embryos, Sim-1 is expressed at high levels in the ventrolateral third of the dermomyotome in those cells where Pax-3 is up-regulated. At this stage, the expression pattern of Sim-1 exhibits a segmental periodicity that reflects the segmented structure of the dermomyotomes (Fig. 5E). When Sim-1 expression was analyzed in E9.5 splotch embryos, a marked difference in the expression pattern was seen compared to wild-type embryos of the same age. While cells expressing Sim-1 were still present in the somitic mesoderm of splotch embryos, Sim-1-positive cells were no longer segmentally arrayed and instead formed a continuous broad band of cells where the ventrolateral region of dermomyotome is normally found (Fig. 5F). The diffuse expression pattern of Sim-1 in splotch embryos indicates that cells derived from the ventrolateral dermomyotome are still present, albeit in a somewhat disorganized manner. This is consistent with the above results showing cellular organization of the ventral dermomyotome is disrupted in splotch embryos.

**Lateral somite halves from splotch embryos give rise to myoblasts when transplanted into a chick host limb**

While demonstrating a crucial role for Pax-3 in maintaining the epithelial structure of the dermomyotome and in regulating the migration of somitic cells into the limb, the above results do not shed light on whether Pax-3 plays an additional role in the differentiation of limb muscle precursors. To examine whether Pax-3 is also required at later times for the differentiation of limb myoblasts, we have examined whether somites from splotch embryos are capable of responding to signals in the limb that promote muscle differentiation. The lateral half of somites I-III, as well as a lateral piece of the segmental plate, were transplanted into the forelimbs of stage-20 chick embryos. Induction of myogenin was observed when lateral somite halves, but not segmental plate, were transplanted into host chick limbs.

In a series of control experiments we tested whether somites derived from E9.25 wild-type or splotch embryos are capable of undergoing myogenic differentiation when cultured alone in collagen gels. Myogenic differentiation was assayed by looking for transcripts of myogenin in explants, since previous studies have shown that myogenin is not expressed in the segmental plate or in somites I-III (Yee and Rigby, 1993; Kopan et al., 1994). Total RNA prepared from the caudal-most three somites of E9.25 embryos (approximately somites 22-24) was analyzed for expression of myogenin by RT-PCR using oligonucleotides that specifically amplify mouse myogenin sequences (Fig. 6). Transcripts of myogenin were not detected in explants of somites I-III derived from either wild-type or splotch embryos (Fig. 6C, lanes 1 and 2). Furthermore, somites cultured alone in collagen gels for 48 hours did not express myogenin, indicating that cells in the lateral somite require further signals for myogenic differentiation to occur (Fig. 6C, lanes 3 and 4). When lateral somite halves from both wild-type and splotch embryos were transplanted into the forelimb of chick hosts and left for 48 hours to develop, mouse myogenin transcripts were detected in limbs bearing the somite transplants, but not in control unoperated limbs (Fig. 6C, lanes 5-8). Interestingly, similar levels of myogenin expression were seen in limbs bearing wild-type and splotch grafts, indicating that cells in the lateral somite of both wild-type and splotch embryos are equally capable of differentiating into muscle in response to limb-derived signals. Mouse MyoD expression was also found in limbs bearing somite transplants from splotch embryos (data not shown).

To further analyze the differentiation of lateral somite halves derived from splotch embryos, we examined the expression of the mouse myogenin gene in sections through stage-24 chick limbs containing the transplanted splotch somites. Cells derived from the grafted somites were easily distinguished from the host chick tissue by the darker staining of their cell bodies after staining with Giemsa (Fig. 7). In limbs containing somites from splotch embryos, the cells from the graft were highly localized (Fig. 7A,C), and this was in turn reflected in the distribution of myogenin transcripts in limbs bearing a graft. In addition some scattered myogenin-expressing cells were observed near the graft. Silver grains marking the distribution of myogenin transcripts were concentrated over most of the darker Giemsa-stained cells (Fig. 7B,D), indicating that the majority of the mouse somitic cells grafted into the limb are capable of responding to limb-derived signals that induce muscle differentiation.

**DISCUSSION**

In this study we have further defined the role Pax-3 plays in the development of the appendicular muscles in the mouse. Previously it had been shown that Pax-3 is necessary for the development of limb muscles (Franz, 1993; Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994) and that Pax-3 is expressed in migrating limb muscle precursors in the chick (Williams and Ordahl, 1994). The experiments in this paper were directed at establishing (1) whether limb muscle precursors that are derived from the dermomyotome migrate into the limbs of homozygous splotch embryos, and (2) if cells derived from the lateral somite of splotch embryos are able to differentiate into myoblasts when transplanted into a normal limb environment. We have analyzed the migration of presumptive limb muscle precursors in two alleles of splotch, Sp and Sp<sup>2H</sup>, where we failed to observe any migration of somite-derived cells into the forelimb of embryos injected with DiI. This was in stark contrast to wild-type embryos where significant numbers of somite-derived cells colonize the early limb bud (Fig. 2). Although we were unable to undertake successfully a double-labeling of DiI- and Pax-3-labeled cells in the limb bud, a similar distribution of DiI and Pax-3 was seen in E9.5 and E10.5 embryos. Together with the results of Williams and Ordahl (1994), our results indicate that the Pax-3-expressing cells present in the early limb are derived from somites and provide further evidence for limb muscles being derived from somites in mammals (Lee and Sze, 1993).

Our finding that somite-derived limb muscle precursors fail to enter the limb is consistent with earlier studies showing Pax-3-expressing cells are absent from the limbs of splotch embryos (Bober et al., 1994; Goulding et al., 1994). In addition, our results show that Pax-3 acts at an early step in limb muscle precursor cell migration by controlling the expression of c-met in the ventral dermomyotome, a site of strong Pax-3 expression in the embryo. Although our results are consistent with c-met being downstream of Pax-3, it
remains to be determined if Pax-3 acts to regulate c-met transcrip-
tion directly. The observation that c-met is essential for limb muscle precursor migration in the mouse (Bladt et al., 1995), together with our results showing the absence of c-met transcripts in limb muscle precursors in splotch embryos, suggest that the loss of c-met expression may be the principal cause of the limb muscle phenotype in splotch embryos. Pax-3 plays an addition role maintaining the epithelial morphology of cells in the ventral dermomyotome. In splotch embryos, cells in the ventral dermomyotome are disorganized, and as a consequence myogenic precursors that would normally be able to migrate into the limb may be unable to do so. It remains to be determined whether this failure of cell migration is partially attributable to these observed morphological changes or whether the down-regulation of c-met is solely responsible for the cell migration defect.

The observed change to the dermomyotome in homozygous splotch embryos coincides with a change in Pax-7 expression normally seen in maturing somites (Goulding et al., 1994). Pax-7 expression is down-regulated in the ventral dermomyotome by E9.5 in wild-type mouse embryos, a time when Pax-3 expression is normally upregulated in these cells. In splotch embryos a consequence of the loss of Pax-3 expression is that cells in the ventral dermomyotome no longer express a functional Pax-3 or Pax-7 gene. The observation that the loss of Pax-3 function causes cells in the ventral dermomyotome to lose their epithelial morphology is noteworthy in the light of previous studies analyzing the role of the notochord in the pating of the somites (Dietrich et al., 1993; Pourquie et al., 1993; Goulding et al., 1994). Transplantation of an ectopic notochord adjacent to the segmental plate in stage-10 chick embryos leads to the loss of Pax-3 and Pax-7 expression in the dorsal somite (Goulding et al., 1994). These cells subsequently lose their epithelial structure and become a loose mesenchyme that differentiates as sclerotome (Brand-Saberi et al., 1993; Goulding et al., 1994). These results, together with the changes that occur in splotch somites, support a role for Pax-3 and Pax-7 in maintaining the epithelial morphology of the dermomyotome.

Another Pax gene, Pax-2, has also been implicated in mesen-
chymal-epithelial cell morphogenesis. In the developing kidney, expression of Pax-2 is induced in the condensing metanephric mesenchyme as it forms epithelial structures, and as the epithelium matures Pax-2 expression is down-regulated (Dressler et al., 1990). Repression of Pax-2 expression by antisense oligonucleotides prevents the conversion of kidney mesenchyme to epithelium (Rothenpeiler and Dressler, 1993), suggesting that the Pax-2 transcription factor is necessary for this mesenchymal to epithelial transformation. This suggests that proteins regulating epithelial cell morphology may be regulated by a number of Pax genes during embryonic development. Targets for Pax-3 in the dermomyotome could include integrins, cadherins and extracellular matrix proteins or intra-
cellular proteins that mediate cell adhesion, such as β-catenin. One possible target for Pax-3 in somites is N-cadherin, which is expressed in condensing somites and later throughout the dermomyotome (Duband et al., 1987). Expression of N-cadherin in the somites closely correlates with the formation and remodeling of the somite into dermomyotome and sclero-
tome.

The expression of Pax-3 in myogenic precursors indicates that Pax-3 may serve an additional role in the differentiation of limb muscle precursors. Axial muscles and limb muscles are derived from two distinct lineages in the somite (Ordahl and Le Douarin, 1992) and consequently mutations to Pax-3 may affect the differentiation of somitic cells in the lateral somite half that give rise to the appendicular muscles. If this were the case, Pax-3 function would be required first for the migration of limb muscle precursors and then later for muscle differentiation. The observation that axial muscles develop in splotch embryos indicates that the loss of Pax-3 in these cells does not impair their ability to undergo myogenic differentiation. However, given that the Pax-3 and Pax-7 proteins are highly homologous, the continued expression of Pax-7 in the precursors for the axial muscles could compensate for the loss of Pax-3 in these cells. By transplanting lateral half somites from splotch embryos into the wing bud of stage-20 chick embryos, we have shown that the lateral portion of somites from splotch
embryos, when transplanted into the chick limb bud, can generate myoblasts. This finding, together with our results analyzing the migration of limb muscle precursors in splotch embryos, suggests that the loss of limb muscles in splotch embryos occurs solely as a consequence of the lack of muscle precursor migration. The observation that some body wall muscles still develop in splotch mice is consistent with our finding that cells in the lateral half of the somite maintain their potential to undergo myogenic differentiation. While our experiments indicate that the loss of Pax-3 function in the embryo does not inhibit myogenesis, the exact role of Pax-3 in muscle differentiation remains unclear. The observation that Pax-7 is expressed in splotch mice, albeit in a somewhat disorganized fashion, suggests that the activity of Pax-3 or Pax-7 alone may be sufficient for skeletal myogenesis. This question will be best addressed by analyzing myogenesis in Pax-7 null mice and compound homozygous Pax-3/Pax-7 null mice.

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