A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb

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We address the molecular control of myogenesis in progenitor cells derived from the hypaxial somite. Null mutations in Pax3, a key regulator of skeletal muscle formation, lead to cell death in this domain. We have developed a novel allele of Pax3 encoding a Pax3-engrailed fusion protein that acts as a transcriptional repressor. Heterozygote mouse embryos have an attenuated mutant phenotype, with partial conservation of the hypaxial somite and its myogenic derivatives, including some hindlimb muscles. At these sites, expression of Myf5 is compromised, showing that Pax3 acts genetically upstream of this myogenic determination gene.

We have characterized a 145-base-pair (bp) regulatory element, at −57.5 kb from Myf5, that directs transgene expression to the mature somite, notably to myogenic cells of the hypaxial domain that form ventral trunk and limb muscles. A Pax3 consensus site in this sequence binds Pax3 in vitro and in vivo. Multimers of the 145-bp sequence direct transgene expression to sites of Pax3 function, and an assay of its activity in the chick embryo shows Pax3 dependence. Mutation of the Pax3 site abolishes all expression controlled by the 145-bp sequence in transgenic mouse embryos. We conclude that Pax3 directly regulates Myf5 in the hypaxial somite and its derivatives.

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Skeletal muscle formation depends on the myogenic regulatory factors that control muscle cell determination and differentiation [Tajbakhsh and Buckingham 2000]. Myf5 is expressed at the onset of myogenesis in the mouse embryo [Ott et al. 1991] when, together with Mrf4, it determines myogenic cell fate [Kassar-Duchossoy et al. 2004]. Subsequently, MyoD is also expressed and can direct cells into the myogenic program in the absence of Myf5 and Mrf4 [Braun et al. 1992]. In the absence of all three myogenic determination factors, skeletal muscle does not form, and precursor myoblast cells are lacking [Rudnicki et al. 1993; Kassar-Duchossoy et al. 2004].

Regulation of the myogenic determination genes has been extensively studied. As for other genes that control cell fate during development, their transcriptional regulation is complex, reflecting the integration of information required for the fine tuning of skeletal muscle formation at different sites in the embryo. Mrf4 and Myf5 genes are present in the same locus, separated by 5.5 kb of DNA and regulated by 5′ sequences extending over >100 kb [Hadehoul et al. 2000; Carvajal et al. 2001], as well as by elements within the locus [Summerbell et al. 2000]. Many different DNA regions that direct distinct aspects of the spatiotemporal expression of these two genes during myogenesis have been identified. However, little is known about the factors that directly control their activation.

Skeletal muscle in the trunk and limbs derives from somites, segments of paraxial mesoderm that form on either side of the neural tube and notochord, following the anterior/posterior developmental gradient of the embryo [Tajbakhsh and Buckingham 2000]. Myogenic progenitor cells are present in the dorsal epithelium of the somite, the dermomyotome, from which they delaminate to form the skeletal muscle of the myotome, underneath the dermomyotome, or migrate from its hypaxial [ventro–lateral] domain to found muscles such as those.
in the limbs. Activation of the myogenic regulatory genes, leading to myotome formation, depends on signals from the surrounding tissues (Tajbakhsh and Buckingham 2000). An enhancer that is active in the early epaxial (dorso–medial) somite has been characterized within the Msx–Myf5 locus, 3' of the Msx gene (Summerbell et al. 2000). This element is regulated by a Gli-binding site, a potential target for Shh signaling (Gustafsson et al. 2002; Teboul et al. 2003). Part of the epaxial myotome is targeted by another enhancer located at −17 kb from Myf5, and it has been shown that this activity depends on a USF-binding site (Chang et al. 2004). These are the only elements associated with the Msx–Myf5 locus for which any regulatory factors have been described. Other sequences that control the spatiotemporal expression of Myf5 include elements within the gene itself that direct some expression to the early hypaxial dermomyotome and a region adjacent to the promoter that targets myogenic cells in the branchial arches and the face and neck muscles that derive from them (Summerbell et al. 2000). Many of the sites of transcription of Myf5 in the embryo depend on a region located at −58/−48 kb from the gene (Hadchouel et al. 2000, 2003). This is essential for most Myf5 expression in mature somites, notably in the hypaxial dermomyotome and its derivatives including the myogenic cells of the hypoglossal cord and limbs (Buchberger et al. 2003; Hadchouel et al. 2003), where Myf5 and MyoD are expressed once the progenitor cells reach the limb buds (Tajbakhsh and Buckingham 1994).

Genetic data provide insight into the regulatory genes that act upstream of the myogenic factors. The Pax3 and Pax7 genes, encoding paired domain, homeobox factors (Tremblay and Gruss 1994), are important regulators of myogenic progenitor cells. As the somite matures, cells expressing both Pax genes move from the central region of the dermomyotome to the myotome, where they activate Myf5 and MyoD and contribute to the growth of skeletal muscle (Ben-Yair and Kalcheim 2005; Gros et al. 2005; Kassar-Duchossoy et al. 2005; Relaix et al. 2005). In the double Pax3/Pax7 mutant, these cells fail to enter the myogenic program, leading to a major deficit in skeletal muscle (Relaix et al. 2005). Pax3, unlike Pax7 in the mouse embryo, is expressed at the extremities of the epithelial dermomyotome, notably in the hypaxial dermomyotome, where it plays an important role in ensuring the survival of myogenic progenitor cells (Borycki et al. 1999). In Pax3 mutants this domain is lost, leading to the absence of muscles, such as those in the limb, formed by progenitor cells that migrate from the hypaxial dermomyotome (Tajbakhsh and Buckingham 2000). A gain-of-function allele, expressing Pax3–FKHR, a strong transcriptional activator, rescues the Pax3 mutant phenotype, thus demonstrating that Pax3 acts as a transcriptional activator during myogenesis. This is confirmed by the expression of the P34 transgene where the reporter is regulated by Pax3-binding sites (Relaix et al. 2003). At the onset of myogenesis, skeletal muscle formation depends on the delamination of cells from the edges of the dermomyotome, where Pax3, Myf5, and Mrf4 are expressed. In the triple mutant, no skeletal muscle forms and MyoD is not activated (Tajbakhsh et al. 1997; Kassar-Duchossoy et al. 2004), whereas in the presence of Pax3 or Myf5/Mrf4, myogenesis occurs. This led to the conclusion that Myf5 and Mrf4 act independently of Pax3 in the genetic hierarchy that regulates the initiation of skeletal muscle formation. However, although epaxial myogenesis proceeds under the control of Myf5 in the absence of Pax3, loss of the hypaxial dermomyotome, due to cell death in the Pax3 mutant (Borycki et al. 1999), precludes any analysis of the regulatory relationship between these genes in this domain.

We have now developed a novel Pax3 allele that expresses a Pax3–Engrailed fusion protein, which we had previously shown behaves as a dominant-negative form of Pax3 in cultured muscle cells (Relaix et al. 2006). Heterozygote Pax3Pax3–En–IRESnlacZ/+ embryos have a hypomorph-like phenotype in which the dermomyotome and its derivatives are partly conserved, in contrast to Pax3 mutants. In myogenic cells in the hypaxial somite, hypoglossal cord, and limb buds, Myf5 expression is severely compromised. Direct activation of Myf5 by Pax3 is demonstrated by the characterization of a 145-base-pair (bp) element within the −58/−48 regulatory region, 5' to the Myf5 gene. This directs transgene expression to the mature somite, notably to the hypaxial dermomyotome and its derivatives, including myogenic cells in the limb. This element binds Pax3 in vivo, and the Pax3-binding site is essential for its activity. We therefore conclude that at these locations Pax3 can act directly upstream of Myf5. Together with the genetic evidence provided by the Pax3Pax3–En–IRESnlacZ/+ allele our analysis demonstrates that this novel genetic hierarchy is implicated in the regulation of hypaxial myogenesis.

**Results**

**Heterozygote embryos expressing the Pax3–En repressor have a hypomorph-like phenotype**

We have targeted the Pax3 gene with a sequence encoding a form of Pax3 that acts as a transcriptional repressor, Pax3–En [Fig. 1A]. This hybrid transcription factor contains at its N-terminal the Pax3 DNA-binding domains fused to the repression domain of the Drosophila Engrailed transcriptional repressor (Han and Manley 1993). When overexpressed in satellite cell cultures, such a fusion protein acts as a dominant-negative form of Pax3 (Relaix et al. 2006). When the Pax3GFP(Pax3–En–IRESnlacZ) allele [Fig. 1A] is crossed with an ubiquitously expressing PGK-Cre mouse (Lallemand et al. 1998) to remove the floxed GFP-FRT–Puro cassette, it generates the Pax3Pax3–En–IRESnlacZ/+ allele. At birth, no living heterozygotes were obtained, demonstrating that expression of the Pax3–En fusion protein leads to embryonic lethality. Introduction of an IRESnlacZ reporter following the Pax3–En sequence makes it possible to visualize β-galactosidase (β-gal)-positive cells expressing this allele, allowing comparison with heterozygote embryos in which one allele of Pax3 is targeted with nlacZ (Pax3nlacZ/+).
Relaix et al. 2004] and with mutant embryos in which there is a second null allele (Pax3 nlacZ/Sp). Analysis of Pax3Pax3–En-IRESnlacZ/+ embryos suggests that they have a hypomorph-like phenotype. This phenotype is not due to lack or reduced expression of the other Pax3 allele as revealed by in situ hybridization (data not shown). Pax3Pax3–En-IRESnlacZ/+ embryos (Fig. 1D) usually show no exencephaly and reduced spina bifida, characteristic features of Pax3 mutants (Fig. 1C). At embryonic day 11.5 (E11.5), Pax3 mutant embryos lack myogenic cells in the forming limb buds or hypoglossal cord, which gives rise to throat and tongue muscles (Fig. 1C). In contrast, these cells are detectable in the hypoglossal cord and in hindlimb, but not in forelimb buds, of Pax3Pax3–En-IRESnlacZ/+ embryos (Fig. 1D), indicating that migration of myogenic progenitor cells from the hypaxial somite is only partially compromised. The presence of myogenic cells in the forming muscle masses of the hindlimb, although reduced, is also seen at E12.5 (Fig. 1G, H9252), and at later stages (data not shown), whereas the forelimb bud remains negative (Fig. 1G), as in Pax3 mutant embryos (Fig. 1F). In the absence of Pax3, somite abnormalities, with foreshortening of the epaxial and, notably, the hypaxial domains of the dermomyotome, are more pronounced than in Pax3Pax3–En-IRESnlacZ/+ embryos (Fig. 1B–D). By E11.5, the myotome in the Pax3 mutant has begun to recover (Fig. 1C), with the contribution of progenitor cells that normally express both

Figure 1. The Pax3Pax3–En-IRESnlacZ allele: strategy and phenotypes. (A) General strategy of Pax3–En-IRESnlacZ targeting into the Pax3 locus. (Pax3–En) cDNA sequence encoding the Pax3–Engrailed fusion protein; (IRES) internal ribosome entry site; [nlacZ] reporter encoding β-galactosidase [β-gal] with a nuclear localization sequence [n]; [GFP] green fluorescent protein; [Puro pA] puromycin selection cassette; [loxP] target sites for Cre recombinase; [FRT] target for Flip recombinase. [B–G] X-gal staining of Pax3nlacZ/+ control embryos (B, E, E), Pax3–En–IRESnlacZ/+ (Pax3–En+/+) embryos where one allele encodes the dominant-negative factor Pax3–En [D, G, G] at E11.5 [B–D] and forelimbs (outlined) [E–G] and hindlimbs [E–G] at E12.5. [B–D] Black arrowheads indicate the hypoglossal cord; white arrows show the normal localization of hindlimb muscle masses. (H–J) View of the thoracic somites, stained by X-gal, of Pax3nlacZ/+ [H], Pax3Pax3–IRESnlacZ/+ [I], and Pax3Pax3–En–IRESnlacZ/+ [J] embryos at E10.5. [K–M] Coimmunohistochemistry on transverse sections (level of section boxed in H–J) of thoracic somites showing the hypaxial region of the dermomyotome Pax3nlacZ/+ [K], Pax3Pax3–IRESnlacZ/+ [L], and Pax3Pax3–En–IRESnlacZ/+ [M] embryos at E10.5, using antibodies against β-gal [Pax3, red] and activated caspase 3, which marks dying cells [green]. Nuclei are shown by DAPI staining [blue].
Pax3 and Pax7 from the remaining central part of the dermomyotome. At E10.5, truncation of the extremities of the dermomyotome, with complete loss of the hypaxial domain, is striking in the absence of Pax3 (Fig. 1I), whereas in the presence of Pax3–En, most of the cells in the hypaxial domain are maintained (Fig. 1J). This is also shown by coimmunohistochemistry on sections of thoracic somites, where cell death, detected by expression of activated caspase 3, is evident in this domain in the mutant only [Fig. 1K–M]. We therefore conclude that the presence of Pax3–En does not lead to extensive loss of myogenic progenitor cells in the hypaxial somite, although the migration of these cells to the limb buds is affected. The presence of Pax3-expressing cells in the hypaxial somite and hindlimb bud in Pax3Pax3–En-IRESnlacZ/+ embryos enables us to investigate the effects of interference with Pax3 function on the entry of cells into the myogenic program in these domains.

Expression of Myf5 is reduced in the hypaxial dermomyotome of Pax3Pax3–En-IRESnlacZ/+ embryos

A comparison of Myf5 transcripts by in situ hybridization at E9.5 shows that in Pax3Pax3–En-IRESnlacZ/+ embryos (Fig. 2D), Myf5 transcripts are not detected in the hypaxial somite compared with the control (Fig. 2C), despite the maintenance of this domain in the presence of Pax3–En (Fig. 2B). These results suggest that Pax3 is required for hypaxial initiation of Myf5 expression. This is also evident at E10.5 when there is a major deficit of Myf5 transcripts in the hypaxial domain (Fig. 2H), com-

Figure 2. Impaired hypaxial Myf5 expression in the presence of Pax3–En. [A,B] X-gal staining of Pax3nlacZ/+ [A] and Pax3Pax3–En-IRESnlacZ/+ [B] embryos at E9.5. [C,D] Whole-mount in situ hybridization using a Myf5 antisense probe on Pax3Pax3–En-IRESnlacZ/+ [C] and Pax3Pax3–En-IRESnlacZ/+ [D] embryos at E9.5. The black arrows point to the hypaxial somite. [E,F] X-gal staining of thoracic somites at E10.5 of Pax3nlacZ/+ [E] and Pax3Pax3–En-IRESnlacZ/+ [F] embryos. [G–N] Whole-mount in situ hybridization of thoracic somites of Pax3Pax3–En-IRESnlacZ/+ [G–M] or Pax3Pax3–En-IRESnlacZ/+ [H–N] embryos at E10.5 using riboprobes recognizing Myf5 [G,H], Mrf4 [I,J], MyoD [K,L], or Sim1 [M,N] transcripts. [O,P] Whole-mount in situ hybridization at forelimb level of Pax3Pax3–En-IRESnlacZ/+ (O) or Pax3Pax3–En-IRESnlacZ/+ (P) embryos at E11.5, hybridized with a riboprobe for c-met transcripts. [Q–S] Coimmunohistochemistry on transverse sections of thoracic somites from Pax3nlacZ/+ [Q], Pax3Pax3–En-IRESnlacZ [R], or Pax3Pax3–En-IRESnlacZ/+ [S] embryos at E10.5, using antibodies recognizing Myf5 [green] and β-gal [red].
pared with the control [Fig. 2G]. Similarly, transcription of Mrf4, present in the same locus as Myf5, is not detectable in this domain [Fig. 2J], where it is normally expressed at this stage [Fig. 2I]. In contrast, MyoD transcripts are still detectable [Fig. 2L], demonstrating the presence of muscle progenitor cells in the hypaxial dermomyotome. Sim1 transcripts, which mark this region [Fan et al. 1996], are also seen in the Pax3Pax3-En-IRESlacZ/+ embryos [Fig. 2N]. We next investigated whether loss of hypaxial Myf5 was also seen at the protein level [Fig. 2Q–S]. Myf5 expression is normally initiated in the Pax3-positive cells at the extremities of the dermomyotome and maintained in these cells, which rapidly down-regulate Pax3 as they move into the myotome [Fig. 2Q]. In Pax3 mutant embryos at E10.5, there is a reduction and disorganization of the dermomyotome, with complete loss of the hypaxial domain. At this stage, a small Myf5-positive myotome is present epaxially [Fig. 2R]. In contrast, in Pax3Pax3-En-IRESlacZ/+ embryos [Fig. 2S], most of the hypaxial dermomyotome is present. Nevertheless, the Myf5 protein is only detected in the epaxial myotome, unlike the control [Fig. 2Q], where it is also expressed hypaxially. These results suggest that Pax3 acts upstream of Myf5 in the hypaxial somite.

Genetic evidence that Pax3 acts upstream of Myf5 at the onset of limb myogenesis

Myf5 is first activated when myogenic progenitor cells, expressing Pax3, reach the limb buds [Tajbakhsh and Buckingham 1994]. In the absence of the tyrosine kinase receptor c-Met, these cells do not delaminate from the hypaxial dermomyotome, and migration from the somite is compromised [Bladt et al. 1995]. In somites of Pax3Pax3-En-IRESlacZ/+ embryos, at E11.5, c-met expression is reduced [Fig. 2P] compared with the control [Fig. 2O], and c-met transcripts are not detectable in the forelimb bud [Fig. 2P]. The presence of Pax3 and Myf5 is shown in Figure 3A–D in a normal hindlimb bud at E11.5, at the onset of Myf5 activation. In contrast to the situation in the myotome (Relaix et al. 2005), many cells (69.4%) coexpress both proteins [Fig. 3E–G,K]. In the presence of Pax3–En, on the other hand, many fewer Myf5-positive cells are detected [Fig. 3H–J]. Fewer Pax3-positive myogenic progenitor cells migrate from the somite [Fig. 1D,G], however, the proportion of these cells that express Myf5 is reduced by ∼50% [Fig. 3K] compared with the control. This is shown at E11.5 for hindlimb buds of Pax3Pax3-En-IRESlacZ/+ , where the X-gal staining for Pax3-positive cells is less intense [Fig. 3M] compared with control embryos [Fig. 3L], but the cells are present and express the myogenic determination gene, MyoD [Fig. 3O]. However, Myf5 expression is severely reduced [Fig. 3Q], compared with the situation in the control embryo where both Myf5 and MyoD are robustly expressed at this stage [Fig. 3N,P]. This result suggests that Myf5 activation during limb myogenesis depends, directly or indirectly, on Pax3.
Identification of a 145-bp element that directs Myf5 expression in the limb

Deletion of the −59/−54 region 5’ to the mouse Myf5 gene, in the context of a BAC containing 195 kb of genomic DNA including the Mrf4–Myf5 locus, had already shown that this region is essential for most expression of Myf5, targeted with \( nlacZ \), in the limb buds, as well as in the hypoglossal cord and in the mature somites. Dissection of regulatory sequences within this region led to the identification of a 500-bp sequence, at −57.5/−57 kb upstream of Myf5, that is responsible for expression in the hypoglossal cord and limb buds as well as in part of the myotome and in the hypaxial dermomyotome (Hadchouel et al. 2003). A 270-bp sequence, which overlaps with the 500-bp sequence, had been reported to have similar activity (Buchberger et al. 2003). We therefore isolated the 145-bp fragment that is common to both sequences, and introduced it upstream of an \( nlacZ \) reporter cassette containing 3 kb of 5’ flanking sequence, including the Myf5 promoter and branchial arch (ba) regulatory element (Summerbell et al. 2000), which acts as a positive control (Fig. 4A). The expression profile of this transgene was examined both in a transgenic line (Fig. 4B–E) and in transient transgenic embryos (Fig. 4F–H; see Table 1). Figure 4I shows the complete Myf5 expression profile.

![Figure 4](genesdev.cshlp.org). A 145-bp element, located at −57.5 kb upstream of Myf5, directs expression in somites, trunk, and limb muscles. (A) Schematic representation of the 145 baMyf5nlacZ transgene. A 145-bp element located at −57.5 kb from the Myf5 gene was cloned upstream of a 3-kb fragment that includes the Myf5 promoter and a branchial arch (ba) element as an internal control (not drawn to scale). (B–E, G, H) Whole-mount X-gal staining of 145 baMyf5nlacZ transgenic embryos at E10.5 (B), E11.5 (C, G), E12.5 (D), E13.5 (H), and E15.5 (E). (I) Whole-mount X-gal staining of a control embryo at E13.5 expressing a y240Myf5nlacZ transgene that recapitulates the complete Myf5 expression pattern. (F) Section of a thoracic somite from a transgenic embryo at E11.5, showing labeled myogenic progenitor cells in the epithelial structure of the hypaxial dermomyotome (also inset), as well as scattered \( \beta \)-gal+ cells, in the myotome (bracket). White arrowheads indicate the hypaxial domain of the somite (C, G) or one of its derivatives: the rectus abdominis muscle (D, E, H, I). Black arrowheads point to distal limb muscles, present in the control embryo (I), but lacking in D, E, and H. The gray arrowheads in B, C, and G point to the hypoglossal cord. Labeling in the neural tube (B) as well as the branchial arches is due to the Myf5 promoter region (Summerbell et al. 2000), present in the transgenes. In the transgenic line, ectopic expression in dorsal root ganglia is also seen. [ba] Branchial arches, [fl] forelimb, [hl] hindlimb. B–E are from a transgenic mouse line; C and G are from the y240Myf5nlacZ line (Hadchouel et al. 2000).
expression pattern at E13.5, directed by a YAC transgene carrying 240 kb of genomic DNA extending 5′ from the Myf5 gene [Hadchouel et al. 2000]. Prior to E10.5 (30 somites), the only myogenic expression observed with the 145baMyf5nlacZ transgene is in the branchial arches, due to the ba element (data not shown). Expression in the ventral neural tube in transgenic embryos is probably due to a sequence within this ~3-kb Myf5 flanking region [Summerbell et al. 2000]. Other sites of transgene expression are first detected in myogenic cells in the forelimb at E10.5 [Fig. 4B]. Weak staining is also seen in the hypoglossal cord, which gives rise to tongue and throat muscles. Like those of the limbs, these myogenic cells derive from the hypaxial dermomyotome of somites at the corresponding axial level. At E11.5 [Fig. 4C,G], the 145baMyf5nlacZ transgene directs expression in the developing fore- and hindlimbs, as well as the hypoglossal cord. By this stage, transgene expression is also detected, with variable intensity, in somites, notably, in the thoracic region, in the epithelial structure of the hypaxial dermomyotome (Fig. 2F), as well as in scattered myogenic cells in the myotome. Myogenic cells present in the hypaxial dermomyotome at this stage contribute to the formation of major ventral trunk muscles. At E12.5 [Fig. 4D], E13.5 [Fig. 4H], and E15.5 [Fig. 4E], labeling of these muscles, including the rectus abdominus, is observed. Some epaxial muscle derivatives, located dorsally, are also labeled by the transgene, reflecting expression in some cells of the epaxial myotome. At fetal stages, 145baMyf5nlacZ is expressed in many of the developing limb muscles [Fig. 4D,E,H], although not in the most distal muscles, in contrast to the control embryo [Fig. 4I]. The 145-bp element therefore directs transgene expression to several important myogenic sites from E11.5. It is particularly active in derivatives of the hypaxial somite, including many abdominal muscles and muscles of the limbs.

Identification of a functional Pax3-binding site in the 145-bp sequence

The sequence of the 145-bp fragment is shown in Figure 5A. It is highly conserved between human, mouse, and chick genomes, suggesting that this noncoding sequence has an essential regulatory function. Within the conserved 145-bp sequence, there is a putative Pax3-binding site, AGGCATGACT, with 70% homology with a previously described consensus sequence [Epstein et al. 1996]. We verified that Pax3 can bind to this site by using a His-tagged purified Pax3 protein in an electrophoretic mobility shift assay with part of the 145-bp sequence, including the putative Pax3-binding site [Fig. 5C]. Specificity of binding was shown by competition with increasing concentrations of cold probe. No competition was observed with a probe in which the Pax3 site was mutated (ΔPax3) [see Fig. 5B]. The main DNA–protein complex is supershifted in the presence of an antibody to Pax3, but not by an antibody to Pax7 [Fig. 5C]. We therefore conclude that the 145-bp fragment contains a Pax3-binding site.

In order to verify that Pax3 binds to this sequence in vivo, we performed chromatin immunoprecipitation (ChIP) experiments on nuclear extracts from E11.5 embryos. As a control, to check that the Pax3 antibody works under these conditions, we prepared extracts from embryos of the P34 transgenic line, in which multimers of the consensus Pax3-binding site direct transgene expression to sites of Pax activity [Relaix et al. 2004]. The fold amplification by PCR of the multimerized consensus Pax3-binding site (P34) compared with an unrelated control sequence, X3, [Navarro et al. 2005] demonstrates that the Pax3 antibody selectively immunoprecipitates chromatin to which Pax3 is bound [Fig. 5D]. Similar extracts prepared from wild-type embryos showed enrichment of the 145-bp sequence after immunoprecipitation of chromatin with the Pax3 antibody [Fig. 5D] but not with a control antibody [NG2] [Fig. 5D′]. We also observed enrichment of the 145-bp fragment compared with sequences lying 5′ or 3′ to it [Fig. 5D′]. In the experiments shown in Figure 5, D and D′, whole-embryo extracts were used after removal of the head and internal organs. Immunoprecipitation with the Pax3 antibody also gave enrichment of the 145-bp sequence with extracts from limb buds or from myogenic progenitor cells isolated by flow cytometry from the limb buds of Pax3GFP/+ embryos [Montarras et al. 2005]. The small quantities of these cells, many of which have activated Myf5 [Fig. 3D], led to high background levels that tended

| Injected constructs | Number of transitory transgenic embryos showing myogenic expression/number of transgenic embryos | Number of transgenic lines showing myogenic expression/number of transgenic lines |
|--------------------|-------------------------------------------------|-----------------------------------------------|
| −58/−57 baMyf5nlacZ | 7/7                                              |                                               |
| −58/−57 tknlacZ    | 4/4                                              |                                               |
| −58/−57ΔPax3 baMyf5nlacZ | 1*/10                             |                                               |
| −58/−57ΔPax3 tknlacZ | 0/3                                              |                                               |
| 145 baMyf5nlacZ    | 10/13                                            |                                               |
| [145]X6 tknlacZ    | 3/3                                              |                                               |
| [145]X2 tknlacZ    | 3/3                                              |                                               |

*One embryo showed extensive ectopic expression, with some blue cells at sites of myogenesis.
to negate the advantage of dealing with a pure population. However, a threefold enrichment was seen (data not shown), confirming that Pax3 binds the 145-bp sequence in vivo, in myogenic cells in the limb.

**Demonstration of the functional significance of the Pax3-binding site**

Previous experiments with the P34 mouse line had shown that a concatenated consensus Pax3-binding site can direct reporter gene transcription to all sites of Pax3 expression in transgenic embryos (Relaix et al. 2003, 2004). In order to examine Pax3 function in the context of the 145-bp sequence, we introduced concatemerized consensus Pax3-binding sites (tk) into transgenic embryos [without head and internal organs] at E11.5. Histograms indicate the fold enrichment with the 145-bp sequence (145), compared with an unrelated control sequence, X3 (Navarro et al. 2005), after ChIP with a Pax3 antibody. P34 embryos, which express a transgene regulated by Pax3-binding sites [Relaix et al. 2004], provide a positive control [P34]. Results are the average of three PCR assays per sample, in two independent experiments. (D') Example of a ChIP experiment showing the binding of Pax3 (black) or of a control NG2 antibody (gray) expressed as the percent of DNA immunoprecipitated, using primers for the 145-bp sequence (145), for control sequences at 200 kb 5' (5') or at 2.3 kb 3' (3') from it, and for the X3 sequence.
labeled, and at later fetal stages, weak expression of (145)tknlacZ is still detectable in limbs and abdominal muscles (data not shown). When fewer copies of the 145-bp sequence are present, the expression pattern begins to resemble that of Myf5 rather than Pax3, such that with two copies of the 145-bp sequence, which give easily detectable transgene expression, the tknlacZ transgene is mainly expressed at sites of myogenesis, with non-myogenic Pax3-like expression retained in the dorsal root ganglia, but not in the trigeminal ganglia or in the neural tube (Fig. 6C). These results point to the importance of the Pax3-binding site in vivo as a target for Pax3 activation and reveal the dominant effect of this interaction when multiple sites are present. This suggests that other factors, which prevent activation of the 145-bp sequence at nonmyogenic sites of Pax3 expression, are progressively titrated out as the number of copies of this sequence increases, or that, in the presence of Pax3 binding to multiple sites, the requirement for other myogenic coactivators is overcome.

Further indication of the functional importance of the Pax3-binding site comes from experiments in the chick embryo (see Table 2). Electroporation into the neural tube of a −58/−57 baMyf5lacZ transgene, which includes the 145-bp element (Fig. 4A), led to low level expression of β-gal (Fig. 6D,E), probably due to the presence of Pax3 in the dorsal neural tube. However, when a Pax3 expression vector is coelectroporated, β-gal levels are much higher (Fig. 6F,G), whereas this expression is lost when the Pax3-binding site in the 145-bp sequence is mutated (Fig. 6H). These results suggest that other factors, which prevent activation of the 145-bp sequence at nonmyogenic sites of Pax3 expression, are progressively titrated out as the number of copies of this sequence increases, or that, in the presence of Pax3 binding to multiple sites, the requirement for other myogenic coactivators is overcome.

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Pax3 activates Myf5 during myogenesis

mutated [Fig. 6H,I]. These experiments therefore show that Pax3 transactivates the transgene through the Pax3-binding site.

The importance of this site for expression in the mouse embryo is demonstrated by transgenic experiments with the −58/−57 fragment in which the Pax3-binding site is mutated. This was used in preference to the 145-bp sequence alone, which is more subject to integration site effects and gives less robust expression. As shown previously (Hadchouel et al. 2003), the 1-kb fragment has a similar expression profile, with pronounced activity in the hypaxial dermomyotome and limb buds when present in a −58/−57 baMyf5nlacZ transgene [Fig. 6J]. Confirmation that this activity resides in the −58/−57 fragment is provided by the expression profile obtained with a transgene in which the proximal 3 kb of Myf5 flanking sequence was replaced by the nonmyogenic thymidine kinase [tk] promoter. X-gal staining is similar, with additional ectopic expression in the posterior edge of the limb buds [Fig. 6L]. When the Pax3-binding site in the 145-bp sequence is mutated in the context of both of these transgenic constructs, myogenic activity in the somites and limb buds is lost [Fig. 6K,M]. With the baMyf5 promoter, in addition to some expression in anterior muscles derived from the branchial arches and in the neural tube, weak residual labeling at the base of the limb buds is observed, probably due to sequences in the promoter [Fig. 6K, data not shown]. With the tk promoter, no labeling is detectable when the Pax3 site is mutated, except for ectopic β-gal activity in a few cells in the head [Fig. 6M]. We therefore conclude that the Pax3-binding site is essential for the activity of the 145-bp sequence in vivo.

Discussion

We have shown genetically that Pax3 acts upstream of Myf5 in the hypaxial somite and its derivatives, including myogenic cells in the limb. Analysis of a Myf5 regulatory element that directs expression of this myogenic determination gene in mature somites and in the limb buds shows that its activity depends on a site that binds Pax3 in vivo, thus demonstrating direct regulation of Myf5 by Pax3.

A novel genetic approach to Pax3 function

We had previously developed a gain-of-function allele of Pax3 that expresses PAX3–FKHR, a fusion protein in which the DNA-binding sequences of Pax3 are followed by the strong transcriptional activation domain of FKHR (FOXO1A) [Relaix et al. 2003]. Pax3PAX3–FKHR–IRESnlacZ/Gsp embryos do not show the Pax3 mutant phenotype, consistent with the role of Pax3 as a transcriptional activator during myogenesis. We now show that a Pax3PAX3–En–IRESnlacZ/+ allele encoding a fusion protein, in which the FKHR transactivator sequence is replaced by the Engrailed repression domain, interferes with Pax3 function. The repressor function of Pax3–En had already been demonstrated in muscle satellite cells [Relaix et al. 2006]. Pax3PAX3–En–IRESnlacZ/+ heterozygote embryos have less severe defects than those seen in the Pax3-null mutant. Analysis of this hypomorph phenotype provides novel insight into Pax3 function. This genetic approach is particularly valuable in the case of factors, like Pax3, that play a role in cell survival so that cell populations are lost in their absence. In Pax3PAX3–En–IRESnlacZ/+ embryos, we have been able to dissociate different functions of Pax3, and notably its anti-apoptotic role in the myogenic progenitor cells of the hypaxial dermomyotome, which is less affected than its role in the regulation of myogenesis. Other aspects of Pax3 function also show differential effects; the closure of the anterior neural tube, for example, occurs correctly and exencephaly is very rare, whereas spina bifida is observed more frequently. Pax3 from the wild-type allele is present together with the Pax3–En protein, which probably competes for Pax3 target sites. It may also affect Pax7 targets; however, in the domains of Pax3 expression that are the focus of this paper, namely, the hypaxial somite and early limb bud, Pax7 is not expressed, and Pax3 is therefore the only member of this family present. Pax proteins can function as homodimers [Chalepakis et al. 1994]; however, it is not clear whether Pax3–En retains this potential. Competition between Pax3 and Pax3–En will depend on the relative stabilities of the proteins and on their interaction with the transcriptional apparatus recruited to the Pax3-binding site. Pax3 is a poor transcription factor and probably requires coactivators to be effective [Relaix et al. 2003]. The impact of the Pax3–En transcriptional repressor on Pax3 targets in heterozygote embryos may therefore also depend on the type of cofactors associated with the wild-type Pax3. The Pax3 targets that mediate its anti-apoptotic effect are not yet known. However, our observations on the effects of Pax3–En suggest that such genes may have a higher affinity for the native Pax3 transcriptional complex than Myf5 or Mrf4. Transcription of the c-met gene, which is a direct Pax3 target [Epstein et al. 1996] although reduced, is also less sensitive to the presence of Pax3–En. Different regulatory sequences will also contain sites that recruit other transcription factors that may modulate Pax3/Pax3–En binding and activity. Differences in the presence of such cofactors and factors may therefore account for differential effects on Pax3 functions in Pax3PAX3–En–IRESnlacZ/+ embryos. The importance of other regulators is illustrated by the observation that multiple copies of the 145-bp sequence direct expression to nonmyogenic sites where Pax3 is present, whereas fewer copies mainly target sites of myogenesis, even with a heterologous promoter. This probably reflects the importance of an equilibrium between the level of Pax3 activity and other regulatory factors necessary for the maintenance of a progenitor state in which myogenic determination factors are not expressed [Relaix et al. 2005].

Pax3 regulation of Myf5 in the somites

In Pax3−En–IRESnlacZ/+ embryos, most of the hypaxial dermomyotome is maintained, and the extensive cell
death seen in the Pax3 mutant is not observed. However, in the presence of Pax3–En, the full hypaxial extent of the dermomyotome is somewhat reduced, and the epaxial structure is compromised. This might suggest that dermomyotome growth, rather than cell survival, is affected, although a low level of apoptosis may not have been detected. Epithelialization of the somite is not essential for the onset of myogenesis, which occurs in the paraxial mutant in the absence of any epithelial structure (Burgess et al. 1996), and, indeed, early epaxial myogenesis takes place in the Pax3-null mutant where the epaxial dermomyotome is affected (Tajbakhsh et al. 1997). The activation of MyoD in the hypaxial domain in Pax3Pax3–En–IRESnLacZ/+ embryos demonstrates that myogenic progenitor cells are present and that the striking absence of Myf5 and Mrf4 expression is not due to the complete loss of these cells in Pax3Pax3–En–IRESnLacZ/+ embryos, also indicated by the maintenance of Sim1 expression. In Pax3Pax3–En–IRESnLacZ/+ embryos, there is not an accumulation of mislocated myogenic progenitor cells, observed in the Myf5/Mrf4 mutant (Tajbakhsh et al. 1996; Kassar-Duchossoy et al. 2004). This may account for the absence of a delay in the activation of MyoD, which is seen in the Myf5/Mrf4 mutant (Tajbakhsh et al. 1997). Myf5 and Mrf4 expression is clearly seen in the epaxial domain in the presence of Pax3–En and the epaxial myotome forms, although, as in the Pax3 mutant, the epithelial structure of the epaxial dermomyotome is affected. This observation points to a distinct regulation of Myf5 and Mrf4 by Pax3 in the hypaxial somite during early myogenesis, as well as at later stages.

The regulation of Mrf4 has not been analyzed in as much detail as Myf5. Although its transcription also depends on distal 5′ sequences (Carvajal et al. 2001), it is not yet clear whether deletion of the −58/−48-kb region, which clearly affects Myf5 (Hadchouel et al. 2003), has as much impact on Mrf4 expression.

The regulatory elements responsible for the early hypaxial transcription of Myf5 probably lie outside the −58/−48-kb region, which contains the 145-bp sequence, since transgene expression in this domain requires more 5′ regulatory sequences (Hadchouel et al. 2000; Carvajal et al. 2001), as well as sequences located within the Myf5 gene (Summerbell et al. 2000). The 145-bp sequence directs later hypaxial expression, which is initiated between E10.5 and E11.5. This is not simply a maintenance effect, since activation of transgene expression is seen with this sequence, unlike a previously described region involved in maintenance of some aspects of Myf5 expression that, alone, was not sufficient for transgene expression (Hadchouel et al. 2000). The 145-bp sequence also directs some later epaxial expression of the transgene, seen not only with the baMyf5 promoter, but also in the presence of the nonmyogenic tk promoter. Early activation of Myf5 in the epaxial dermomyotome depends on the enhancer located at −6.6 kb from the Myf5 gene (Summerbell et al. 2000; Teboul et al. 2002). As at other sites of myogenesis (Hadchouel et al. 2003), the spatiotemporal regulation of Myf5 in the epaxial and hypaxial somite is controlled by different sequences. The 145-bp sequence has a Pax3 consensus site, which binds this factor in vivo and is essential for its activity, demonstrating the direct activation of a Myf5 regulatory element by Pax3. The genetic results presented here suggest that earlier activation of Myf5 in the hypaxial somite also depends on Pax3. The presence of distinct early and late hypaxial elements probably reflects the requirement for different regulatory inputs in addition to Pax3. The evolutionary implications of this are potentially interesting in the context of subpopulations of myogenic progenitor cells in this part of the somite. A classification of muscles has been proposed, based on the mesodermal environment encountered by their progenitor cells (Burke and Nowicki 2003). This distinguishes primaxial myogenic cells that remain within the domain of the somite from abaxial cells that differentiate in the context of lateral mesoderm and the connective tissue derived from it. The 145-bp sequence is not active in the early hypaxial somite, which constitutes a primaxial domain, whereas it is active later in the hypaxial dermomyotome and in the myogenic cells of the ventral body wall muscles and limbs. In this respect, it functions as an abaxial regulatory element, controlling Myf5 in this domain of embryonic patterning.

Pax3 regulation of Myf5 during limb myogenesis

In the presence of Pax3–En, migration of myogenic progenitor cells from the hypaxial somite to the limb buds is compromised. No Pax3-expressing cells are found in the forelimb, whereas they migrate into the hindlimb bud, although in reduced numbers. This may be due to an effect on the c-met gene, known to be a Pax3 target [Epstein et al. 1996; Relaxi et al. 2003]. Transcripts of c-met are reduced in the somites of Pax3Pax3–En–IRESnLacZ/+ embryos, indicating another hypomorphic effect of the Pax3–En allele. Since the c-Met receptor is required for the delamination and migration of myogenic progenitor cells from the hypaxial dermomyotome (Bladt et al. 1995), this might suggest that this process fails to take place if c-Met does not attain a threshold level. It is not clear why some myogenic progenitor cells reach the hind- and not the forelimb. However differences between fore- and hindlimbs have been observed in Lbx1 [Schafer and Braun 1999; Brohmann et al. 2000; Gross et al. 2000] and Meox2 [Mankoo et al. 1999] mutant embryos, where the muscle masses are differently affected by the absence of these factors, which are expressed both in migrating progenitors and in the myogenic cells of the limb. In the case of the Lbx1 mutant, this appears to be due to differential effects on the migration of myogenic progenitor cells to the limbs from the somite [Schafer and Braun 1999; Brohmann et al. 2000; Gross et al. 2000]. Different threshold levels for Pax3 or, indeed, c-Met may be required for the activation of this process at fore- and hindlimb levels, reflecting regulatory differences at different axial levels as development proceeds. Such regulatory differences between fore- and hindlimb have been observed in embryos in which one or two alleles of Pax3
were targeted with a \textit{Pax7} coding sequence [Relaix et al. 2004]. In these embryos, proximal/distal effects on migration into the limb bud have been observed. Transgenes directed by the 145-bp sequence from the \textit{Myf5} locus are not expressed in distal myogenic sites in the limb buds, also reflecting proximal/distal differences in regulatory circuits. Other sequences within the -58/-48 region are also implicated in \textit{Myf5} regulation in the limbs, notably, for example, a 3′ region that directs some transgene expression mainly to the hindlimb bud [Hadchouel et al. 2003]. These may be responsible for the residual \textit{Myf5} expression seen in the hindlimbs of \textit{Pax3−/−} embryos, where many of the remaining muscle progenitor cells are \textit{Myf5} negative. At early stages of myogenesis in the limb buds, the -58/-48 region is essential; however, in the fetus, sequences located elsewhere in the locus direct \textit{Myf5} expression in the limbs [Hadchouel et al. 2003]. The 145-bp sequence is still active at later stages, suggesting that several elements are responsible for the expression of \textit{Myf5} in developing limb muscles.

In the limb bud, unlike the trunk [Relaix et al. 2005], many cells coexpress \textit{Pax3} and \textit{Myf5} as the muscle masses begin to form, perhaps because the entry of muscle progenitor cells into the myogenic program includes an extended “myoblast” compartment, before the cells differentiate. It is probable that in the mouse, as in the chick embryo [Schienda et al. 2006], some of the \textit{Pax3}-positive cells remain as reserve cells and subsequently express \textit{Pax7}, which, unlike the avian embryo [Marcelle et al. 1995], is not initially present in migrating myogenic progenitor cells [Relaix et al. 2004]. Our in vivo ChIP experiments show that \textit{Pax3} binds to the 145-bp sequence and is detected in \textit{Pax3}-expressing cells in the embryonic limb. When the Pax3-binding site is mutated, all transgene expression in the limb buds as well as the somites is lost. Therefore \textit{Pax3} acts directly upstream of \textit{Myf5} during limb myogenesis. Consistent with this, in the \textit{Pax3}−/− embryos, \textit{Myf5} expression is severely down-regulated, whereas \textit{MyoD} transcripts continue to accumulate in both the hindlimbs and hypaxial somite. This is in keeping with observations in the chick limb bud, where expression of \textit{Pax3} and \textit{Myf5}, but not \textit{MyoD}, is affected by Wnt6 manipulation [Geetha-Loganathan et al. 2005]. \textit{Pax3} may therefore not lie genetically upstream of \textit{MyoD} in this limb context and in the hypaxial somite. Alternatively, activation of \textit{MyoD} may require a lower threshold of \textit{Pax3}.

\textit{Pax3} activation and genetic hierarchies

We show here that \textit{Pax3} acts upstream of \textit{Myf5} in the hypaxial domain of the somite. Overexpression of \textit{Pax3} in explants of paraxial mesoderm from the chick embryo led to the up-regulation of both \textit{Myf5} and \textit{MyoD} [Maroto et al. 1997]. It is now clear in the mouse that \textit{Pax3} can lie genetically upstream of both \textit{Myf5} and \textit{MyoD} and that the nature of this hierarchy depends on the myogenic context. Thus, at the onset of myogenesis, analysis of \textit{Pax3} and \textit{Myf5} mutants has shown that \textit{Myf5} is expressed in the absence of \textit{Pax3}, which regulates \textit{MyoD} [Tajbakhsh et al. 1997]. Similarly, in satellite cells, the progenitor cells of postnatal skeletal muscle, expression of \textit{MyoD} depends on \textit{Pax3}/7, whereas \textit{Myf5} is independent of Pax regulation [Relaix et al. 2006]. During late embryonic and fetal myogenesis, on the other hand, \textit{Pax3} and \textit{Pax7}, expressed in progenitor cells in the central domain of the dermomyotome, act genetically upstream of \textit{Myf5} and \textit{MyoD} [Relaix et al. 2005]. We therefore conclude that the fine-tuning of myogenesis during development and regeneration is controlled by \textit{Pax3} via the myogenic determination genes \textit{MyoD} and/or \textit{Myf5}, according to the spatiotemporal context. Furthermore, we have now shown that transcription of \textit{Myf5}, in abaxial muscle progenitor cells, is regulated by a sequence that is directly activated by \textit{Pax3}.

\textbf{Materials and methods}

\textbf{Targeting vectors and the generation of embryos with a \textit{Pax3}\textsuperscript{−}\textit{En}\textsuperscript{−}\textit{IRES}\textsuperscript{−}\textit{nlacZ} allele}

The targeting construct is derived from one that was previously reported (Relaix et al. 2004). Cloning details are available on request. Briefly, the \textit{Pax3}\textsuperscript{−}\textit{En}\textsuperscript{−}\textit{IRES}\textsuperscript{−}\textit{nlacZ} allele contains 2.4 kb of the 5′ \textit{Pax3} genomic region, replacing the coding sequence of exons 1 and 4 kb of 3′ sequence containing exons 2–4. The genomic sequences surround a floxed \textit{GFP}\textsuperscript{-}\textit{FRT}\textsuperscript{−}\textit{Puro} cassette followed by a \textit{Pax3}\textsuperscript{−}\textit{En} fusion sequence, which encodes mouse \textit{Pax3}, amino acids 1–374, fused with \textit{Drosophila} Engrailed, amino acids 1–298. This is followed by an \textit{IRES}\textsuperscript{−}\textit{nlacZ} sequence with an \textit{FRT} site inserted between the \textit{nlacZ} and the \textit{pA} sequence (into the Xbal site of the pSK\textsuperscript{−}\textit{nlacZ} plasmid) [Relaix et al. 2004]. In addition, a \textit{PGK}\textsuperscript{−}\textit{DTA} cassette encoding the A subunit of the \textit{Dipteria toxin} gene [Meilhac et al. 2003] was inserted 5′ of the constructs to allow negative selection in embryonic stem (ES) cells [not shown in Fig. 1A]. The targeting vector was electroporated into CK35 ES cells [Kress et al. 1998]. ES cells were selected and screened for recombination events by Southern blot analysis using EcoRV digests and a 5′ probe and verified using 3′ and internal probes shown in Fig. 1A. Targeted ES cells were recovered with a 0.5%–1% frequency, and two clones were injected into blastocysts to generate chimeras. Germline transmitted alleles were identified by the classical \textit{Splotch} heterozygote phenotype [lack of melanocyte colonization of the belly], and by PCR or by Southern blotting. Two independent ES clones gave germline transmission and the same phenotypes. These mice expressed GFP (Montarras et al. 2005). After removal of the \textit{GFP} and \textit{ParapA} cassettes, which are surrounded by Lox-P sites, by crossing with PGK\textsuperscript{Cre} mice [Lallemand et al. 1998] expressing the Cre recombinase, embryos expressing \textit{Pax3}\textsuperscript{−}\textit{En}\textsuperscript{−}\textit{IRES}\textsuperscript{−}\textit{nlacZ} were generated.

\textbf{Plasmid constructions used for transgenesis}

To generate the 145\textsuperscript{ba}\textit{Myf5}\textsuperscript{−}\textit{nlacZ} transgene, the 145-bp element was synthesized by PCR using Advantage 2 Taq polymerase (Clontech) with Xhol and HindIII sites added to 5′ and 3′ primers, respectively. The forward and reverse primers used were f\textsuperscript{\textit{wd}} (5′-AAACATCGAGTATGTCCATATTCTTCCC-3′) and r\textsuperscript{\textit{rev}} (5′-AAAAAGGACTTAAATGTCCCCATATTCTTCCC-3′). After sequence verification, the PCR product was cloned into a pGEM-T Easy vector [Promega], followed
by cloning of the 145-bp Xhol–HindIII fragment into the baMyf5nlacZ plasmid [Hadchouel et al. 2000]. The [14S]6tknlacZ transgene was generated by the procedure described in Relaix et al. [2003]. The forward and reverse primers used were fwd (5′-AAAGGCCACGCTCGCCGTTTGTGGAAGGCC-3′) and rev (5′-AAAGGCCACCTGGCCAAATTGCCATATTA CCTCCC-3′). The -58/-57 baMyf5nlacZ transgene was as described previously [Hadchouel et al. 2003]. Mutagenesis was performed with the QuickChange Site-Directed Mutagenesis kit [Stratagene], using as a matrix a plasmid in which the -58/-57 fragment had been subcloned into a pGEM-T Easy vector [Promega]. The -58/-57 Pax3 fragment was released from the pGEM-T Easy vector by HindIII/Xhol or NotI digestion and subcloned into baMyf5nlacZ or into tknlacZ plasmids [Hadchouel et al. 2003], respectively. The primer used for mutagenesis was 5′-CATAAAGGACGTCTAATTTGCTGTAACGTG-3′. The 58/-57 tknlacZ transgene was generated by cloning the -58/-57 NotI fragment into the tknlacZ vector [Hadchouel et al. 2003].

**Generation of transgenic mice**

Plasmid fragment purification was carried out as described [Kelly et al. 1995]. Transgenic mice were generated as described previously [Hadchouel et al. 2000]. Transient transgenic embryos were dated taking E0.5 as the day after the appearance of the vaginal plug. For X-gal staining, embryos dissected in PBS were fixed for 10–45 min (depending on the stage) with 4% paraformaldehyde (PFA) in PBS, on ice. Embryos were rinsed twice with PBS, then stained with X-gal (Roche) for 4–45 min (depending on the stage) with 4% paraformaldehyde (PFA) in PBS, on ice. Embryos were rinsed in PBS and dated taking E0.5 as the day after the appearance of the vaginal plug. For X-gal staining, embryos dissected in PBS were fixed for 10–45 min (depending on the stage) with 4% paraformaldehyde (PFA) in PBS, on ice. Embryos were rinsed twice with PBS, then stained with X-gal [Roche] for 4–16 h at 37°C, with shaking [Relaix et al. 2003]. Embryos were rinsed in PBS and post-fixed overnight in 4% PFA. Whole-mount in situ hybridization with digoxigenin-labeled probes for MyoD was performed as described in Tajbaksh et al. [1997]. The e-met probe was a gift from C. Birchmeier [Berlin, Germany] [Bladt et al. 1995]. The mouse Str1 probe was a gift from C.-M. Fan [Baltimore, MD] [Fan et al. 1996]. Fluorescent coimmunohistochemistry on sections was carried out as described previously [Relaix et al. 2003]. The following antibodies were used: anti-Mys6, 1:200 [Daubas et al. 2000] or 1:100 [Santa Cruz]; monoclonal anti-β-galactosidase, 1:200 [Sigma]; monoclonal anti-Pax3, 1:50 [kindly provided by M. Bronner-Fraser, Pasadena, CA]; and polyclonal anti-active caspase 3, 1:250 [PharMingen]. Secondary antibodies were coupled to a fluorochrome, Alexa 488, 546, or 594 [Molecular Probes], and used at dilutions of 1:250 [Alexa 488] or 1:1500 [Alexa 546 or 594]. Images were obtained with Aportome Zeiss and Axiosview software. This system provides an optical section view reconstructed from fluorescent samples, using a series of “grid projection” or “structured illumination” acquisitions. Figures were assembled using Photoshop CS [Adobe] with a PowerMac G4 and an iMac G5.

**Electricophoretic mobility shift assay (EMSA)**

An oligonucleotide containing the Pax3-binding site (5′-AAT CATAAAGGCACTGATAATGCATGG-3′) was radiolabeled with γ-32P and then annealed with the cold complementary oligonucleotide to give a labeled probe. The Pax3 coding sequence was subcloned as a cDNA into a pET30a expression vector [Novagen, Inc.] to give a his-tagged Pax3 protein, induced in bacteria, and purified on a Ni-NTA column following the standard conditions [Qiagen]. His-tagged Pax3 protein was incubated with the labeled probe in a reaction mixture containing 75 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl (pH 7.5), 6% glycerol, 2.5 µg of bovine serum albumin, 1 µM of poly(dI-dC) and, in competition experiments, with variable amounts of cold DNA probe either wild type or with the CAT GACT sequence replaced by ACGTCTA. For supershift experiments, 1 µL of mouse monoclonal Pax3 antibody [provided by M. Bonner-Fraser] or mouse monoclonal Pax7 antibody [Developmental Studies Hybridoma Bank] was added to the DNA–protein complex. DNA–protein complexes were separated on 5% polyacrylamide gels in 0.25× TBE buffer [Molinari et al. 2004].

**ChIP**

Embryos at E11.5 were used for ChIP experiments. In the results shown in Figure 5D, the heads and internal organs were removed from wild-type or P34 transgenic embryos [Relaix et al. 2004]. Further experiments were carried out with extracts from limb buds and also with cells separated by flow cytometry from the limb buds of Pax3GFP/+ embryos [Montarras et al. 2005]. The ChIP procedure was adapted from the method previously described [Molinar et al. 2004]. Dissociated cells were fixed with 1% formaldehyde at room temperature for 15 min. Cells were frozen in lysis buffer at −80°C. Nuclei were sonicated for 19 pulses of 10 sec to obtain DNA fragments comprising between 2000 and 2000 bp. Rabbit polyclonal Pax3 antibody [ActiveMotif] and, as a negative control, rabbit polyclonal NG2 antibody [Chemicon Int.] were used at 1:200 dilution. The immunoprecipitated [IP] DNA and the input [IN] DNA were analyzed by real-time PCR using a SYBR Green Universal Mix [Applied Biosystems] and an ABI Prism 7700 [Perkin Elmer Applied Biosystems]. The sequences of the primers used to amplify the 145-bp sequence were fwd (5′-AGGAAGAGCTTGATGGACCAA-3′) and rev (5′-CTCATATAATGATGTTTTAAGCCCC-3′). Primers used as a negative control to amplify the X3 sequence were as described previously [Navarro et al. 2005]. Primers for sequences lying 200 kb 5′ or 2.3 kb 3′ to the 145-bp fragment were as follows: 5′ fwd (5′-TTGTGTGACTGATGATACTAG-3′), 5′ rev (5′-AGGAAGAGCTTGATGGACCAA-3′), 3′ fwd (5′-GTATCCGCTTACTGATGATACTAG-3′), 3′ rev (5′-ACCCTGTTAGAGATTACCAAC-3′). Each PCR assay was run in triplicate, and the percentage of immunoprecipitation was calculated by dividing the average amount of IP by the average amount of IN.

**In ovo electroporation into the chick neural tube**

Fertilized chicken eggs (Société française de production agricole) were incubated at 38°C until embryos reached the Hamburger-Hamilton (HH) stage 11–13. Myf5 plasmid preparations contained 0.2% Fast Green dye [Sigma], for visualization during injection. A reporter plasmid, pCIG [Megason and McMahon 2002] expressing GFP, was included in the solution at 0.5–1 µg/µL to monitor the efficiency of electroporation. The complete mouse Pax3 cDNA was cloned into the multiple cloning...
site of the pCIG plasmid for transactivation studies. The DNA solution was injected by mouth with a drawn-out glass capillary (GC100-T; Harvard Apparatus) into the neural tube at the caudomедullary junction and electroporated into half of the neural tube with a BTX ElectroSquarePorator ECM 830 (Genetronics) configured to deliver five 50-msec pulses of 25 V at a frequency of 1 Hz across the embryo, through a pair of gold-plated electrodes 5 mm in length and 0.5 mm in diameter (Genetrons Model 512, Genetronics, Inc.). Two milliliters of Hank’s balanced salt solution (Sigma) containing penicillin–streptomycin was injected by mouth with a drawn-out glass capillary solution as previously described. Table 2 summarizes the behavior of reinjected embryos. After 1 d of reincubation, GFP activity was visualized using a Zeiss Stemi SV11 UV binocular microscope (using Nikon ACT-l image capture software). Living embryos showing a GFP-positive neural tube were harvested, dissected, rinsed in PBS, and fixed in 4% PFA for 15 min. Embryos were stained overnight at 37°C in X-gal solution as previously described. Table 2 summarizes plasmid expression of GFP-positive electroporated embryos.

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