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Differential regulation of epaxial and hypaxial muscle development by Paraxis

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SUMMARY

In vertebrates, skeletal muscle is derived from progenitor cell populations located in the epithelial dermomyotome compartment of the each somite. These cells become committed to the myogenic lineage upon delamination from the dorsomedial and dorsolateral lips of the dermomyotome and entry into the myotome or dispersal into the periphery. Paraxis is a developmentally regulated transcription factor that is required to direct and maintain the epithelial characteristic of the dermomyotome. Therefore, we hypothesized that Paraxis acts as an important regulator of early events in myogenesis. Expression of the muscle-specific *myogenin-lacZ* transgene was used to examine the formation of the myotome in the *paraxis*$^{-/-}$ background. Two distinct types of defects were observed that mirrored the different origins of myoblasts in the myotome. In the medial myotome, where the expression of the myogenic factor Myf5 is required for commitment of myoblasts, the migration pattern of committed myoblasts was altered in the absence of Paraxis. In contrast, in the lateral myotome and migratory somitic cells, which require the expression of *MyoD*, expression of the *myogenin-lacZ* transgene was delayed by several days. This delay correlated with an absence of *MyoD* expression in these regions, indicating that Paraxis is required for commitment of cells from the dorsolateral dermomyotome to the myogenic lineage. In *paraxis*$^{-/-}$*/myf5*$^{-/-}$ neonates, dramatic losses were observed in the epaxial and hypaxial trunk muscles that are proximal to the vertebrae in the compound mutant, but not those at the ventral midline or the non-segmented muscles of the limb and tongue. In this genetic background, myoblasts derived from the medial (epaxial) myotome are not present to compensate for deficiencies of the lateral (hypaxial) myotome. Our data demonstrate that Paraxis is an important regulator of a subset of the myogenic progenitor cells from the dorsolateral dermomyotome that are fated to form the non-migratory hypaxial muscles.

Key words: Paraxis, Dermomyotome, Myotome, Muscle, Hypaxial, Mouse

INTRODUCTION

Somites establish the segmental body plan of vertebrates by giving rise to vertebrae, ribs, cartilage, skeletal muscle and the dermis of the back, as well as imposing a metameric pattern on peripheral nerves and vascular primordia. Each somite forms as a spherical epithelial ball, derived from the paraxial mesoderm at the anterior end of the segmental plates (for review see Christ and Ordahl, 1995). The somite matures into three mesenchymal compartments which will adopt chondrogenic, myogenic or dermal cell fates. This process begins with the formation of the sclerotome by the de-epithelization of the ventral half of the somite in response to signals from the notochord. The myotome then forms by the delamination of cells from the remaining epithelial structure referred to as the dermomyotome. This compartment is the anlagen for most of the skeletal muscle in the body. The remaining dermomyotome then dissociates into mesenchyme and contributes to the dermal layer.

Early myogenesis is governed by a complex set of morphological and migratory events which begin with the colonization of the medial myotome by cells originating from the dorsomedial lip of the dermomyotome (Ordahl and Le Douarin, 1992; Denetclaw et al., 1997). These cells migrate in a ventrolateral direction and elongate both rostrally and caudally, resulting in a parallel array of myocytes with centrally located nuclei. Myoblasts from the medial half of the myotome are fated to form the epaxial muscles, which make up the deep muscles of the back. The process is duplicated at the dorsolateral lip of the dermomyotome, except that the cells enter the lateral edge of the myotome and migrate medially. Cells from the medial and lateral myotome eventually meet, resulting in a continuous sheet of myoblasts. The lateral myotome then expands ventrally, penetrating the somatopleure of the lateral mesoderm to give rise to the hypaxial muscles of the body wall, including the segmented intercostal muscles, the abdominal wall muscle (internal and external obliques and transverse abdominis) and the rectus abdominis at the ventral midline. In the occipital and cervical somites, myogenic progenitor cells from the dorsolateral lip of the dermomyotome will also migrate to populate the muscle primordia of the hypoglossal muscles of the tongue (Noden, 1983) and the diaphragm (Bladt et al., 1995), and into the limb bud to form the girdle and appendicular muscles (Christ and Ordahl, 1995).

Commitment and differentiation of cells to the muscle lineage requires the expression of a family of basic helix-loop-helix

(bHLH) transcription factors (MyoD, Myf5, Myogenin and MRF4). During embryonic development, the myogenic bHLH genes are expressed in an overlapping pattern in the myotome (reviewed in Buckingham, 1992). *myf5* mRNA transcripts are first detected in cells at the medial edge of the myotome at embryonic day 8 (E8.0; Ott et al., 1991) and expression spreads ventrolaterally with the expansion of the myotome. *myogenin* (*Myog)* is transcribed next in the myotome at E8.5, followed by the transient expression of *MRF4* (E9.0 and E11.5) (Sassoon et al., 1989; Hinterberger et al., 1991; Bober et al., 1991). *MyoD* is expressed last at E10.0. In the somites of the trunk, the pattern of *MyoD* expression is distinct from the other bHLH factors in that it is highest in the myoblasts at the lateral edge of the myotome. Transcription of the myogenic bHLH factors in the forelimb buds is delayed until E10.0, when *myf5* is initially detected, and this is rapidly followed by *MyoD* and *myogenin*.

Gene knockout experiments have revealed that Myf5 and MyoD possess redundant functions and are required for the commitment of cells to the myogenic lineage. Mice deficient in either *Myf5* or *MyoD* have the normal array of muscles, while mice lacking both genes are devoid of cells expressing muscle-specific genes (Braun et al., 1992; Rudnicki et al., 1992, 1993). There is evidence to suggest that these genes initially define two independently regulated myoblast cell populations (Braun et al., 1994; Braun and Arnold, 1996; Kablar et al., 1997). This has led to a model of cellular redundancy, in which *Myf5*-dependent medial and *MyoD*-dependent lateral myoblast populations are able to expand and to compensate for one another in response to deficiencies in the commitment of cells to the myogenic lineage.

The myogenic progenitor cells from the dermomyotome that migrate distally to the limbs and other unsegmented muscles undergo an additional level of regulation. These cells must reliably migrate to the site of the muscle primordia while retaining their mitotic potential, allowing for the expansion of this cell population at remote sites in the embryo. Expression of the paired-homeobox domain protein Pax3 in the dermomyotome and migratory cells has been shown to be important for commitment to the myogenic lineage through the regulation of MyoD transcription, migration and proliferation (Franz et al., 1993; Tajbakhsh et al., 1997; Maroto et al., 1997; Amthor et al., 1998, 1999). Additionally, interaction between c-Met, a cell surface receptor tyrosine kinase expressed in cells fated to migrate, and its ligand, hepatocyte growth factor (HGF) expressed in the lateral mesoderm of the limb field, are required for migration (Bladt et al., 1995, Heymann et al., 1996, Dietrich et al., 1999).

Another candidate for regulating premyogenic events in the dermomyotome is the developmentally regulated bHLH transcription factor, Paraxis (Burgess et al., 1995; Sosic et al., 1997; Barnes et al., 1997). In response to signals from the surface ectoderm, *paraxis* is transcribed in the segmental plate and the newly forming somites. As the somite compartmentalizes, *paraxis* expression becomes restricted to the cells of the dermomyotome. Mouse embryos deficient for Paraxis failed to form proper somites. Mesoderm from the segmental plate faithfully formed segmental units; however, the cells failed to become arranged in an epithelial ball. This results in patterning defects in the tissues derived from these somites. In the axial skeleton, dual sites of ossification occurred within individual vertebra, as well as fusions between the vertebrae and ribs. The vertebral column was truncated below the twenty second vertebra. Muscle defects were also apparent including reduction of the intercostal muscles from three layers to a single hypertrophic layer and a loss of the transverse abdominis muscle (Burgess et al., 1996). Considering that *paraxis* expression is downregulated in the sclerotome and myotome prior to cell fate specification, these observations point to a role for the gene in the specification and/or migration of the premyogenic and prechondrogenic cell populations.

In this study, we examined the role of Paraxis in regulating the initial events in the establishment of the epaxial and hypaxial myotome. The tight correlation between the epithelial state of the myogenic precursor cells of the dermomyotome and their migration into the myotome suggests that the muscle phenotype of the *paraxis*$^{-/-}$ mice may be due to a deregulation of this process. To test this, we generated mice that were mutant or wild type at the *paraxis* locus and carried a *myogenin-lacZ* transgene. We observed that Paraxis was not required for the commitment of myoblasts to the epaxial myotome at E9.5, but was necessary for appropriate migration and organization. In contrast, the formation of the lateral myotome was delayed by several days in the mutant embryos. In the *paraxis*-null background, neonatal epaxial and appendicular muscles are normal, suggesting that the defects observed during embryonic myogenesis do not affect the formation of these muscles. We hypothesized that Myf5-dependent myoblasts from the epaxial myotome are able to compensate for the early deficiencies in the hypaxial myotome. To test this, mice were generated that carried null mutations in both *paraxis* and *myf5*. These experiments revealed a differential requirement for Paraxis in the development of non-migratory hypaxial muscle.

MATERIALS AND METHODS

Intercrosses and genotyping

The *paraxis* mutant mice were described previously (Burgess et al., 1996). Mice carrying mutations in this gene were identified by Southern blot analysis by probing *Sac*I-digested genomic DNA with a 300 bp *Eco*RI-*Sac*I fragment (Burgess et al., 1996). The *myf5* mutant allele was generated by the insertion of a PGKneo cassette into the first exon, upstream of the bHLH domain (Braun et al., 1992). The mutant *myf5* allele was identified by Southern blot by digesting genomic DNA with *Bam*HI and probing with a 340 bp *Nco*I-*Kpn*I fragment from the 3′ end of the first exon. The probe hybridizes to a 9 kb fragment from the wild-type allele and a 6 kb fragment in the mutant allele. Mice carrying the *myogenin-lacZ* transgene were described previously (Cheng et al., 1995). The presence of the transgene was determined by Southern blot by probing *Sac*I-digested genomic DNA with a *lacZ*-specific probe.

Histology and β-galactosidase staining

The expression of the *myogenin-lacZ* transgene was detected by β-galactosidase activity as described previously (Cheng et al., 1995). Briefly, mothers were killed and the embryos dissected away from the uterus. The extraembryonic membranes were removed and saved as a source of genomic DNA for Southern blot analysis. Embryos were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde, in phosphate-buffered saline (PBS) and then stained in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; SIGMA) in PBS, overnight at room temperature. Prior to histological analysis, the embryos were postfixed in 4% paraformaldehyde for an additional 24 hours.

The preparation of embryonic and neonatal tissue for histological analysis was performed as described previously (Rawls et al., 1998). Neonates were skinned and eviscerated prior to fixation in 4% paraformaldehyde in PBS overnight at 4°C. The tissue was embedded in paraffin after a stepwise dehydration in ethanol and xylene. In embryos previously stained with X-gal, ethanol was substituted with methanol to avoid a reduction in stain intensity. 7 μm transverse sections were generated, deparaffinized in xylene and either stained with Hematoxylin and Eosin or analyzed directly.

Immunostaining was performed as described.

Reverse transcription-PCR

Total cellular RNA was isolated from E11.5 embryos using TRIzol (Gibco-BRL), as described in Rawls et al. (1995). The detection of MyoD, Myogenin, Pax1 and L7 mRNA by PCR was performed in a semiquantitative manner as described by Münsterberg et al. (1995) and as modified by Rawls et al. (1995). The number of cycles required to detect the PCR product in the linear range of amplification was determined for each primer pair. Primers for detecting gene-specific transcripts were: MyoD forward primer 5′-AGGACACGACTGCTTTCTTC-3′ and reverse primer 5′-GCACCGCAGTAGAGAAGTGT-3′ resulting in a 389 bp product (27 cycles); myogenin forward primer 5′-CTGGGGACCCCTGAGCATTG-3′ and reverse primer 5′-ATCGCGCTCCTCCTGGTTGA-3′ resulting in a 272 bp product (27 cycles); and L7 forward primer 5′-GGAGCTCATCTATGAGAAGGC-3′ and reverse primer 5′-AAGACGAAGGAGCTGCAGAAC-3′ resulting in a 202 bp product (20 cycles).

In situ hybridization

The embryonic pattern of transcription was determined by whole-mount in situ hybridization using antisense RNA-labelled digoxigenin-11-UTP probes as described by Molkentin et al. (1997). E9.5 embryos were probed with antisense RNA specific to a 340 bp *Nco*I-*Kpn*I fragment from the first exon of *myf5*. E10.5 embryos were probed for the transcription of *MyoD* (Sassoon et al., 1989) and *Pax3* (Goulding et al., 1993).

Cell proliferation assay

In vivo cell proliferation was determined by the incorporation of the thymidine analog, BrdU, into the DNA of actively dividing cells. A cocktail [10 mM BrdU (Sigma) and 1 mM fluorodeoxyuridine (Sigma)] was injected intraperitoneally (0.01 ml/gram of body weight) into pregnant mothers 30 minutes before killing. Embryos were fixed overnight in 4% paraformaldehyde and embedded in Paraplast-plus (Oxford). Transverse 8 μm sections were analyzed for the incorporation of BrdU into cells using a cell proliferation immunoassay (Zymed). Tissue sections were deparaffinized, hydrated through a graded series of ethanol washes and endogenous peroxidase activity quenched by treating with a 9:1 methanol:H_2O_2 solution. To enhance the immunoreactivity of the BrdU, tissue sections were boiled for 10 minutes in a 10 mM citrate buffer, pH 6.0 (Zymed). Application of the BrdU-specific antibody and the subsequent color reaction were performed as described by Fukuda et al. (1990). Sections were counterstained with Hematoxylin and the incorporation of BrdU in the cells of the dermomyotome were counted and photomicrographs were taken at ×20 magnification.

RESULTS

Analysis of medial myotome development in *paraxis*$^{-/-}$ embryos using the *myogenin-lacZ* transgene

The myogenic progenitor cells of the dermomyotome are maintained as part of an epithelial sheet prior to migration into the myotome. It is not known if the transition of these cells to mesenchyme regulates the onset of migration into the epaxial myotome. In *paraxis*$^{-/-}$ embryos, the somites fail to adopt an epithelial state, therefore we wanted to examine the temporal and spatial regulation of myotome formation in these mutants. We chose to address this question by crossing a transgenic mouse line that directs transcription of *β-galactosidase* (*lacZ*) from the *myogenin* enhancer/promoter (*myogenin-lacZ*; Cheng et al., 1995) into the *paraxis*$^{-/-}$ background. The transgene is initially expressed at E9.0 in committed myoblasts of the rostral myotome and is maintained in all skeletal muscle throughout fetal development. The appearance of the myotome as determined by expression of the *myogenin-lacZ* transgene, occurs at a reproducible rate in a rostral-to-caudal direction. Therefore, the rate of onset can be determined by comparing the caudal extent of myotome formation between mutant and wild-type littermates. If progenitor cells in the *paraxis* mutant embryos enter the myotome prematurely more somites will express the transgene. Conversely, a delay in migration would result in fewer somites expressing the transgene.

Mice heterozygous for *paraxis* and carrying the *myogenin-lacZ* transgene were bred. The offspring were harvested between E9.5 and E12.0, after which time most somites have formed a myotome. Expression of the transgene was visualized in whole embryos by a colorimeteric assay. At all embryonic ages examined, there was no significant difference in the number of somites that contained β-gal-positive cells when *paraxis*-null and wild-type littermates were compared (Table 1). This suggests that the rate at which cells migrate out of the dorsomedial lip and their commitment to the myogenic lineage does not require Paraxis.

Even though there was no quantitative difference in the number of somites with a β-gal-positive myotome in the presence versus absence of Paraxis, qualitative differences were evident. In the wild-type background, β-gal-positive cells were restricted to the region of the myotome directly subjacent to the dorsomedial lip of the dermomyotome of E9.5 embryos (20 somites) (Fig. 1A). As the myotome matured, the myoblasts became centrally aligned and migrated in a ventrolateral direction. Elongation of the differentiating myocytes to the rostral and caudal limits of the myotome could be visualized with the expression of the *myogenin-lacZ* transgene, since the β-galactosidase protein is localized in both the cytoplasm and the nucleus (Fig. 1C,E). *Paraxis*$^{-/-}$ embryos

Table 1. The absence of *Paraxis* does not affect the onset of medial myotome formation

Estimated embryonic age	*Paraxis* genotype	Total number of somites	β-gal-positive somites
E9.0 to E9.5	+/+	20	6
	+/+	21	6
	+/+	22	18
	+/+	27	23
	–/–	18	7
	–/–	20	7
	–/–	22	18
	–/–	27	23
E12.0	+/+	52	44
	–/–	51	44

The number of somites positive for expression of the *myogenin/lacZ* transgene was determined in *paraxis*$^{-/-}$ and *paraxis*$^{+/+}$ littermates at the embryonic ages indicated.

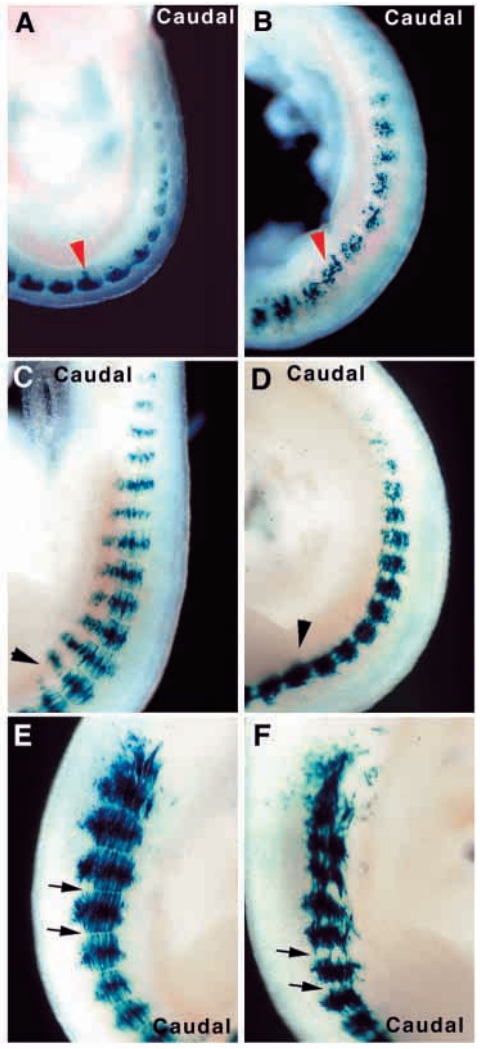

Fig. 1. The spatial organization of the myotome is lost in *paraxis*$^{-/-}$ embryos. Expression of the *myogenin-lacZ* transgene was visualized by X-gal staining in 20- and 27-somite stage wild-type (A,C,E) and *paraxis*$^{-/-}$ (B,D,F) mouse embryos. The migration of *myogenin-lacZ*-positive myoblasts was restricted to the medial boundary of the myotome in the 20-somite stage wild-type embryo (A) but have spread ventrolaterally in the *paraxis*$^{-/-}$ embryo (B). The red arrowhead denotes the myoblasts of a single somite. In 27-somite stage embryos, ventral migration of myoblasts has been initiated in the forelimb level (black arrowhead) of the wild-type (C) but not the *paraxis*$^{-/-}$ embryo (D). The organization of myocytes in a parallel fashion extending from the rostral-to-caudal margins of the myotome can be visualized in the cervical somites of a 27-somite stage embryos (E). The myocytes in the *paraxis*$^{-/-}$ embryos are deficient for both elongation and orientation (F). The black arrow denotes the intersomitic boundaries.

failed to recapitulate the wild-type pattern of myotome development. In 20-somite embryos, the medial edge of the myotome was truncated, and β-gal-positive myoblasts were scattered randomly (Fig. 1B). A similar phenotype was observed in the caudalmost myotome of 27-somite embryos, though the degree of scatter appeared reduced. In addition, the myocytes failed to span the myotome, nor were they consistently oriented along the rostral-caudal axis (Fig. 1D,F). These results point to a role for Paraxis in both the pattern of myoblast migration in the medial myotome, and the organization of myocyte differentiation.

Analysis of ventral myotome formation and the hypaxial muscle in *paraxis*$^{-/-}$ embryos

Defects in the intercostal and abdominal wall muscles of the *paraxis*$^{-/-}$ neonates suggest that this gene is required for the appropriate formation of the hypaxial myotome of the thoracic and lumbar somites that give rise to these muscles. The *myogenin-lacZ* transgene was used to visualize the hypaxial muscles between E9.5 and E13.5 in *paraxis*$^{-/-}$ embryos. In wild-type embryos, the hypaxial myotome could be distinguished in the thoracic somites of at E9.5 as a distinct population of myoblasts that migrated ventrolaterally, but had not begun to elongate (Fig. 1C). By E11.5, the myotome of the thoracic and abdominal somites have invaded the somatopleure (dorsal lateral mesoderm), extending towards the ventral midline (Fig. 2A,C). At E13.5, the abdominal region has divided into the ventrally located rectus abdominus and the unsegmented muscles of the abdominal wall (Fig. 2G). In the *paraxis*$^{-/-}$ embryos, the ventrolateral migration of β-gal-positive myoblasts was absent at E9.5 (Fig. 1D) and E10.5 (data not shown). By E11.5, the *myogenin-lacZ*-positive cells of the myotome have extended ventrally into the lateral plate mesoderm in the mutant embryos, though not to the same degree as the wild-type littermates (Fig. 2B). Interestingly, the growing ventral edge of the myotome is bifurcated in the mutant embryos (Fig. 2D), suggesting that migration of these cells is not regulated by the same mechanism. Consistent with this, we observed that the intercostal muscles of the *paraxis*$^{-/-}$ embryos at E13.5 have not migrated ventrally to the same extent as in the wild-type embryos, nor was the migration uniform between the segmented units (Fig. 2H). Similarly, deficiencies were present in the muscles of the abdominal wall, the rectus abdominus muscles were present, though they had not migrated as far ventrally as in wild-type littermates.

We were interested in determining whether the muscles derived from the subpopulation of the dorsolateral dermomyotome that migrate to the limb are also dependent on *paraxis* expression. Myogenic condensations show strong *myogenin-lacZ* transgene expression in the forelimbs and weak expression in the hindlimbs by E11.5 (Fig. 2A,C). However, in the *paraxis*$^{-/-}$ embryos, β-gal-positive cells were not present until E12.5, and then they were present in both the forelimbs and hindlimbs (Fig. 2E,F). The associated migratory muscle primordia of the pectoral girdle were also reduced in these embryos. The influence of Paraxis on the girdles appeared to be restricted to the pectoral muscles, since the myogenic region of the pelvic girdle did not appear to be significantly affected in the mutants. These data indicated that Paraxis plays an important role in the formation of all skeletal muscle derived from the dorsolateral dermomyotome. Similar delays in hypaxial muscle primordia formation have been reported in mice deficient for MyoD (Kablar et al., 1997), suggesting that Paraxis may be required directly or indirectly in *MyoD* expression.

Paraxis is necessary for the normal expression of MyoD in the ventral myotome

To examine whether Paraxis regulates the formation of the

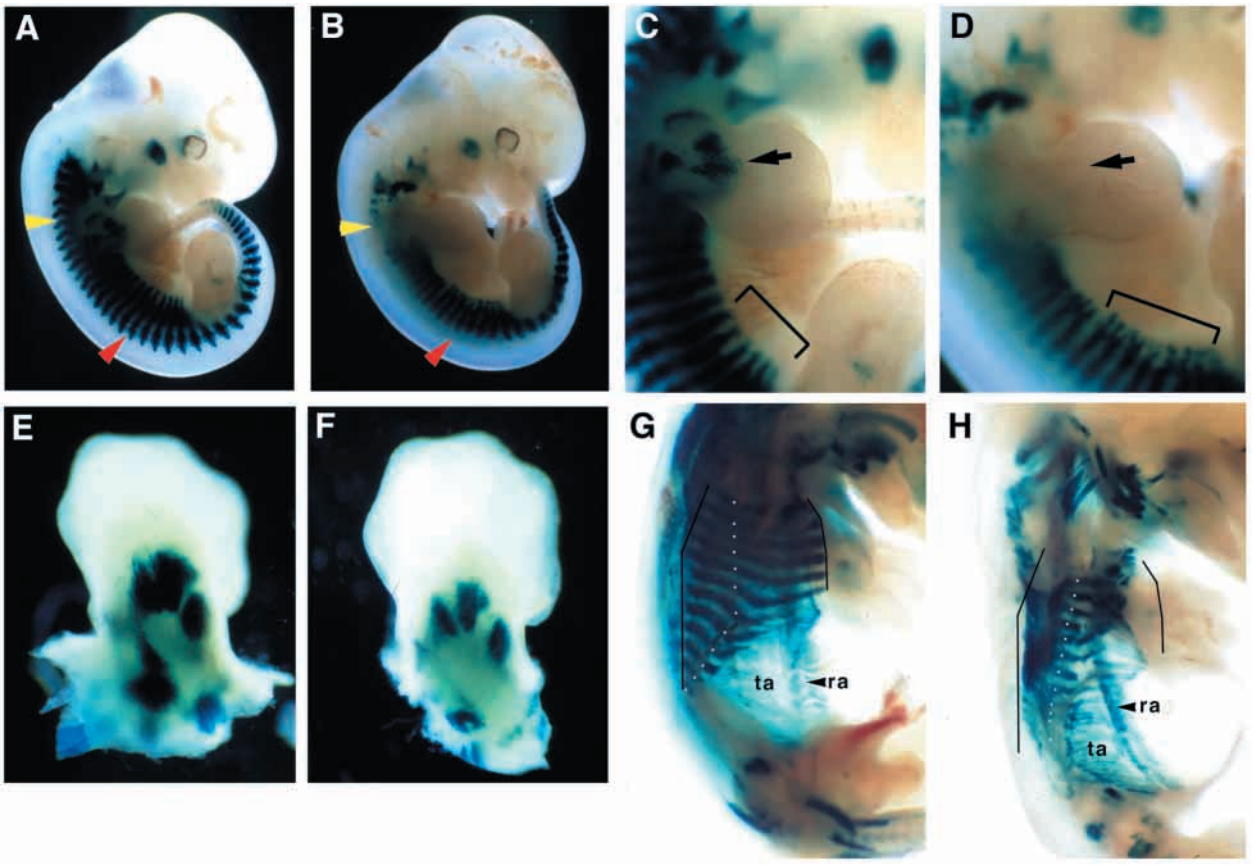

Fig. 2. Formation of hypaxial muscles is delayed in the *paraxis*$^{-/-}$ embryos. Expression of the *myogenin-lacZ* transgene was visualized by X-gal staining in E11.5 (A-D), E12.5 (E,F) and E13.5 (G,H). At E11.5, the myotome and primordia for migratory muscles in the face, forelimb and hindlimb express the *myogenin-lacZ* transgene in wild-type embryos (A,C). In *paraxis*$^{-/-}$ embryos (B,D), the medial myotome is truncated (red arrowhead) and ventral tips of the myotome are bifurcated (bracketed). The primordia for the appendicular migratory muscles are absent in the E11.5 *paraxis*$^{-/-}$ embryos, pectoral girdle (yellow arrowhead) and forelimb (black arrow). *Myogenin-lacZ*-positive cells are present in the forelimbs of both wild-type (E) and *paraxis*$^{-/-}$ (F) embryos at E12.5. In E13.5 wild-type embryos (G), the intercostal muscles have migrated ventrally in a differential manner that reflects the shape of the adjacent developing rib. The corresponding intercostals (white dots) in the *paraxis*$^{-/-}$ embryos (H) fail to migrate ventrally to the same extent. Ra, rectus abdominus; ta, transverse abdominus

MyoD-positive myoblasts derived from the dorsolateral dermomyotome, the transcription of MyoD was determined in E10.5 *paraxis*$^{-/-}$ and wild-type embryos by whole-mount in situ hybridizations. In wild-type embryos, *MyoD* was strongly expressed at the dorsomedial edge of the myotome in cervical somites and the ventrolateral edge of the interlimb somites (Fig. 3A). *MyoD* transcripts were also present in the myotome of the *paraxis*$^{-/-}$ embryos; however, the ventrolateral boundary did not extend as far ventrally as in wild-type littermates (Fig. 3B). This suggested that Paraxis is not required for all *MyoD* transcription, but may be specifically necessary for expression in myoblasts derived from the dorsolateral dermomyotome. This possibility was tested directly by examining *MyoD* transcription in embryos deficient for both Paraxis and Myf5. In the absence of Myf5, myotomal transcription of *MyoD* will be derived solely from the dorsolateral dermomyotome. In E10.5 *paraxis*$^{-/-}$/*myf5*$^{-/-}$ embryos, *MyoD* transcripts were not detectable in the myotome (Fig. 3E,G). MyoD-positive cells were present in the branchial arches, indicating that the loss was specific to the myotome. Consistent with the reported role of Myf5 in regulating early *MyoD* expression in the myotome (Tajbakhsh et al., 1997; Fig. 3C,F), *MyoD* transcription was dramatically reduced but detectable in the *paraxis*$^{+/+}$/*myf5*$^{-/-}$ embryos. In *paraxis*$^{-/-}$/*myf5*$^{+/-}$ embryos, the region of *MyoD* transcription was restricted to a thin band of myoblasts in the medial half of the myotome (Fig. 3D). This pattern was more severe than was observed in the *paraxis*$^{-/-}$/*myf5*$^{+/+}$ embryo, implying haploinsufficiency at the *myf5* gene locus in the absence of Paraxis. These observations are consistent with Paraxis being required of the normal transcription of *MyoD* in the lateral myotome and that its absence is compensated for by Myf5.

MyoD transcription was measured by semiquantitative RT/PCR using total RNA extracts from E11.5 embryos. At this embryonic age, transcription of *MyoD* is not dependent on the expression of *myf5* (Tajbakhsh et al., 1997). Therefore, the examination of embryos from a *paraxis*$^{+/-}$/*myf5*$^{+/-}$ cross at this stage should provide a clearer demonstration of the contribution of Paraxis to *MyoD* expression. As shown in Fig. 4, a greater reduction of MyoD mRNA was observed in the double null embryos when compared to *paraxis*$^{-/-}$ or *myf5*$^{-/-}$ littermates. A similar reduction was observed in the level of *myogenin*, but not the control ribosomal RNA, L7, suggesting the reduction in transcription is specific to muscle lineage. This

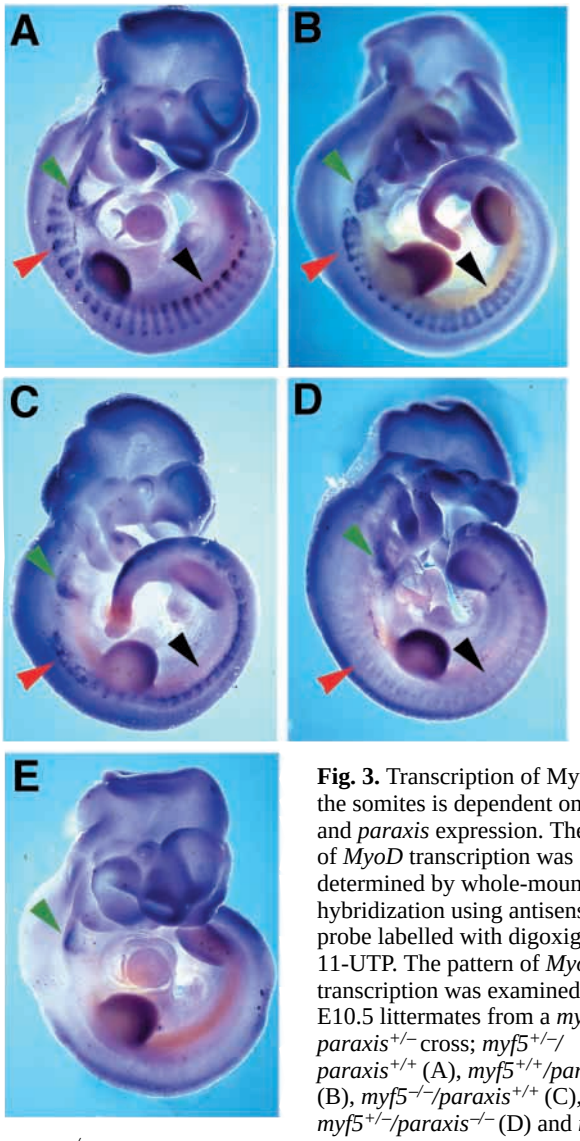

Fig. 3. Transcription of MyoD in the somites is dependent on *myf5* and *paraxis* expression. The pattern of *MyoD* transcription was determined by whole-mount in situ hybridization using antisense RNA probe labelled with digoxigenin-11-UTP. The pattern of *MyoD* transcription was examined in E10.5 littermates from a $myf5^{+/-}$/$paraxis^{+/-}$ cross; $myf5^{+/-}$/$paraxis^{+/+}$ (A), $myf5^{+/+}$/$paraxis^{-/-}$ (B), $myf5^{-/-}$/$paraxis^{+/+}$ (C), $myf5^{+/-}$/$paraxis^{-/-}$ (D) and $myf5^{-/-}$/$paraxis^{-/-}$ (E). The medial and lateral boundaries of *MyoD* transcription in the myotome is denoted by the red and black arrowheads, respectively. The green arrowheads point to MyoD-positive cells in the branchial arches.

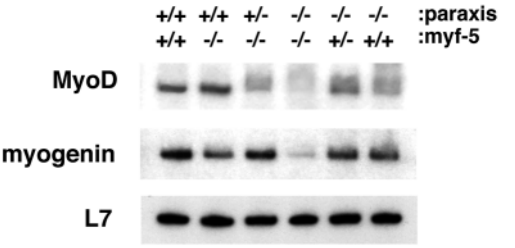

Fig. 4. Analysis of myotome and sclerotome transcripts in wild-type and mutant E11.5 embryos. Total RNA was isolated from the embryos and RT/PCR was performed using primers specific for MyoD, myogenin and L7 as described in Material and Methods.

confirms that *MyoD* transcription in the myotome in the absence of Paraxis is dependent on the activity of Myf5.

MyoD transcription in the somite is dependent on the expression of both Pax3 and Myf5 in all but the occipital somites (Cossu et al., 1996; Tajbakhsh et al., 1997). In the absence of Myf5, *MyoD* transcription is greatly reduced at the time of onset (E10.0) in the myotome but has reached normal levels by E11.5. However, embryos that are deficient for both *myf5* and *Pax3* are devoid of *MyoD* transcripts, implicating *Pax3* in the regulation of *MyoD*. We were interested in determining whether the loss of *MyoD* expression in the $paraxis^{-/-}$ mutant embryos was indirectly due to a loss in the transcription of either *myf5* or *Pax3*. The transcription of these genes was examined in the $paraxis^{-/-}$ embryos by whole-mount in situ hybridization at E9.5 and E10.5. *myf5* transcripts were detectable in an equivalent number of somites in wild-type and *paraxis* mutant embryos. Interestingly, Myf5-positive cells were absent in the lateral myotome of the $paraxis^{-/-}$ embryos, similar to the pattern of expression of the *myogenin-lacZ* transgene (Fig. 5A,B). This would be consistent with a loss of MyoD transcription in the lateral myotome being due to an absence of *myf5* expression in this region of the myotome.

Pax3 is normally expressed in the unsegmented paraxial mesoderm (UPM) and throughout the newly formed epithelial somites. As somites mature, *Pax3* transcription becomes restricted to the dermomyotome, with the highest levels in the dorsomedial and dorsolateral lips (Fig. 5C,E; Goulding et al., 1993; Williams and Ordahl, 1994). The expression of *Pax3* is required for the migration of cells from the dorsoventral edge of the dermomyotome into the appendicular muscle primordia, beginning at E10.0 (Goulding et al., 1997). At E9.5, *Pax3* was transcribed in the UPM and the newly formed caudal somites of the *paraxis* mutant embryos, but not in the maturing somites (Fig. 5D). By E10.5, a narrow band of *Pax3* transcription was detected in the dorsolateral half of the dermomyotome and in the somites between the forelimb and hindlimb (Fig. 5F). *Pax3* transcripts were also present in the proximal region of the forelimb buds of the $paraxis^{-/-}$ embryos, similar to their wild-type littermates though the number of positive cells was significantly reduced (Fig. 5E,F). *Pax3* transcription in the neural tube was equivalent to wild type, suggesting that the differences observed are specific to the somitic mesoderm. It has been previously reported that Pax3 is expressed in the dermomyotomal cells of the caudal somites of the E10.5 $paraxis^{-/-}$ embryo (Burgess et al., 1996). However, a more detailed analysis revealed a more complex picture of *Pax3* regulation by Paraxis. Transcription of *Pax3* initiation is normally in the UPM, but requires Paraxis to be maintained in the dermomyotome. Expression of *Pax3* appears a day later in the limb bud and dorsolateral dermomyotome indicating the presence of a second population of the somitic cells that are regulated independently of Paraxis.

Neonates deficient in *myf5* and *paraxis* fail to form epaxial muscles and a subset of hypaxial muscles

We have demonstrated that Paraxis is required for early events in lateral myotome and hypaxial muscle formation. However, the muscular defects described for $paraxis^{-/-}$ neonates are relatively mild. This could be due to the compensation for these muscle deficiencies by the expression of *myf5* in the epaxial myotome. *Paraxis/myf5* compound heterozygotes were crossed and the litters were allowed to progress to the neonatal stage. All genetic combinations were present at the appropriate Mendelian ratio. The $paraxis^{-/-}$/$myf5^{-/-}$ neonates were similar

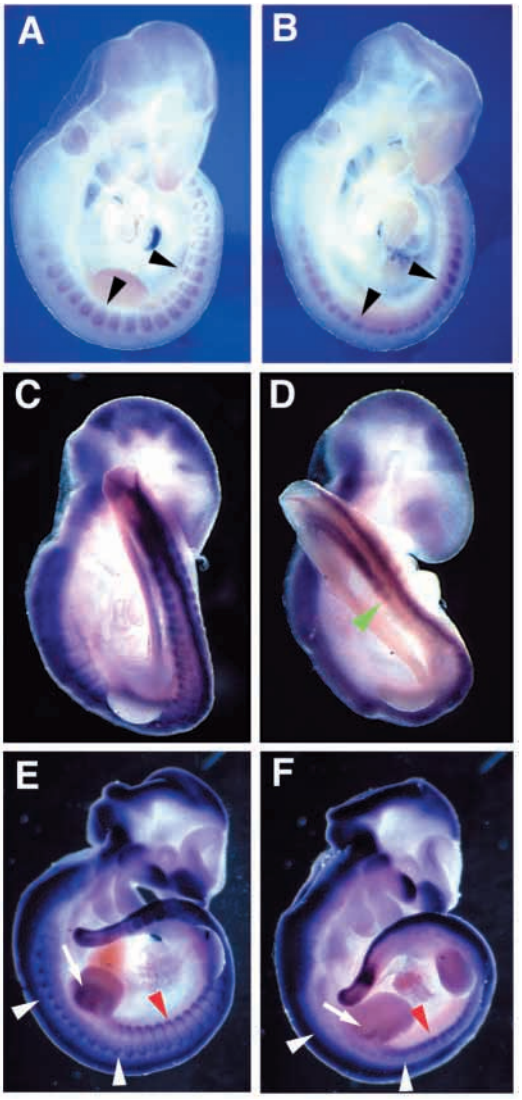

Fig. 5. *Myf5* and *Pax3* are transcribed in the somite of the *paraxis*$^{-/-}$ embryos. The pattern of *myf5* (A,B) and *Pax3* (C-F) transcription was determined by whole-mount in situ hybridization using antisense RNA probes labelled with digoxigenin-11-UTP. *Myf5* transcripts were restricted to the myotome of E9.5 wild-type (A) and *paraxis*$^{-/-}$ (B) embryos. The lateral extent of *myf5* transcription in the thoracic somites is denoted by the black arrowheads. *Pax3* transcripts were detectable in the segmental plate, somites, and dorsal neural tube in the wild-type embryo at E9.5 (C). In contrast, Pax3 was absent rostral to the third somite in the absence of Paraxis (D; green arrowhead). *Pax3* transcripts were detectable in the dorsomedial and dorsolateral lips of the dermomyotome in E10.5 wild-type embryos (E) but only in the dorsolateral lip of the *paraxis*$^{-/-}$ embryos (F). The expression of Pax3 in the dorsomedial and dorsolateral lips of the dermomyotome are denoted by the white and red arrowheads, respectively. The white arrow marks the Pax3-positive cells in the limb muscle primordia.

to the *paraxis*$^{-/-}$/*myf5*$^{+/+}$ littermates in gross morphology, except that the anteroposterior axis was consistently shorter.

Individual muscle groups were examined by histological analysis of transverse thin sections. The epaxial muscles, which consist of the erector spinae, transversospinalis group and interspinalis muscles of the back, originate from the *myf5*-dependent medial region of the myotome (Cossu et al., 1996; Kablar et al., 1997). These muscles are present in both *paraxis*$^{-/-}$ and *myf5*$^{-/-}$ mice, but absent in the *paraxis*$^{-/-}$/*myf5*$^{-/-}$ mice (Fig. 6A-D). In the region between the pelvic and pectoral girdle, these muscles are replaced by loosely packed mesenchyme and, at the thoracic level, the scapula can be seen to lie directly adjacent to the neural arches of the vertebrae. The remaining mesenchymal cells fail to express myosin heavy chain, indicating that they have not entered into the myogenic lineage (Fig. 7). As described above, the *Pax3/MyoD*-dependent myoblasts are able to compensate for the absence of Myf5 in the formation of epaxial muscles. Our results indicate that Paraxis is a necessary component of this compensatory response.

The hypaxial muscles encompass a broader group, which

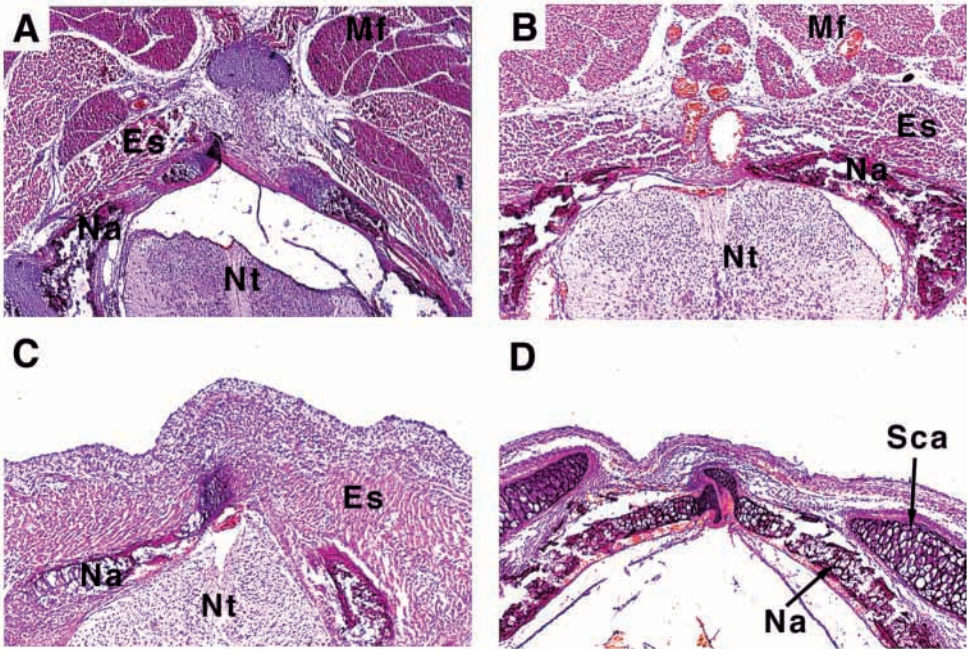

Fig. 6. *Myf5*$^{-/-}$/*paraxis*$^{-/-}$ neonatal mice fail to form paraspinal muscles. Transverse thin sections were cut at the level of the forelimb of wild-type (A), *paraxis*$^{-/-}$ (B), *myf5*$^{-/-}$ (C) and *myf5*$^{-/-}$/*paraxis*$^{-/-}$ (D) neonates and stained with Hematoxylin and Eosin. Deep muscles of the back develop in the absence of either *myf5* or *paraxis*, but not in the compound mutant. Es, Erector Spinae; Na, neural arch; Nt, neural tube; Sca, scapula

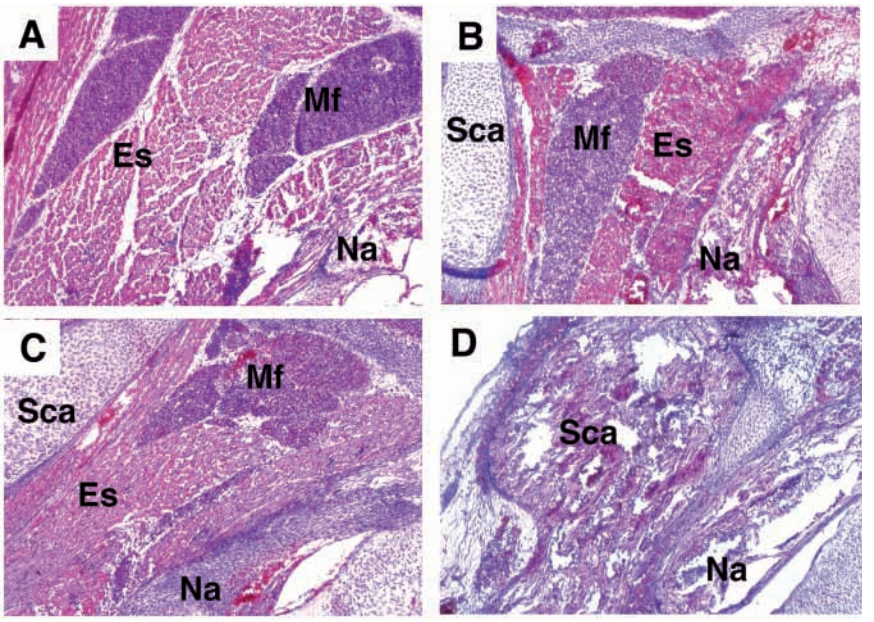

Fig. 7. Myosin Heavy Chain is not expressed in the region of the paraspinal muscles in the absence of Paraxis and Myf5. Transverse thin sections were cut at the level of the forelimb of wild-type (A), *paraxis*$^{-/-}$ (B), *myf5*$^{-/-}$ (C) and *myf5*$^{-/-}$/*paraxis*$^{-/-}$ (D) neonates were immunostained using an antibody specific to the myosin heavy chain and indirectly detected using an anti-mouse antibody conjugated to horse radish peroxidase. The protein was visualized using AEC as a color substrate for the horse radish peroxidase. Es, Erector Spinae; Na, neural arch; Nt, neural tube; Sca, scapula

includes the muscles of the trunk (abdominal wall, intercostals and diaphragm), appendicular (limb, pectoral and pelvic girdles), and muscles of the head and neck (Christ and Ordahl, 1995). Within the trunk of *paraxis*$^{-/-}$/*myf5*$^{-/-}$ neonates, muscles proximal to the vertebral column were affected to a greater extent than those near or at the ventral midline (Fig. 8). The proximal aspect of the external, internal and innermost intercostal muscles were replaced by a single, thin, discontinuous layer of muscle. In contrast, the distal intercostal muscle, which attaches to the sternum, remains at near wild-type levels of muscle mass. Similarly, the proximal abdominal muscles were reduced to a single layer, while the rectus abdominus was not significantly altered (data not shown). The pectoral and pelvic girdles, including the forelimb and hindlimb, also exhibit varying degrees of muscle loss. The supraspinatus and infraspinatus are reduced, but present, in the *paraxis*$^{-/-}$/*myf5*$^{-/-}$ neonates. While the subscapularis on the ventral side and the dorsal muscles that stabilize the scapulae (trapezius, rhomboid major and minor), are completely absent (Fig. 9G). Appendicular muscles were present at approximately half of the muscle mass of *myf5*$^{-/-}$ or *paraxis*$^{-/-}$ littermates, consistent with the reduced expression of the *myogenin-lacZ* transgene in the *paraxis*$^{-/-}$ background. The muscles of the diaphragm, pelvic girdle and tongue in the double mutant are comparable to the *myf5* and *paraxis* single mutations (data not shown). These results indicate that the formation of the hypaxial muscles is not completely dependent on Paraxis. Myoblasts that give rise to proximal (non-migratory) hypaxial muscles are more affected than distal (migratory) hypaxial muscles. An intriguing exception are the muscles at the ventral midline, which are

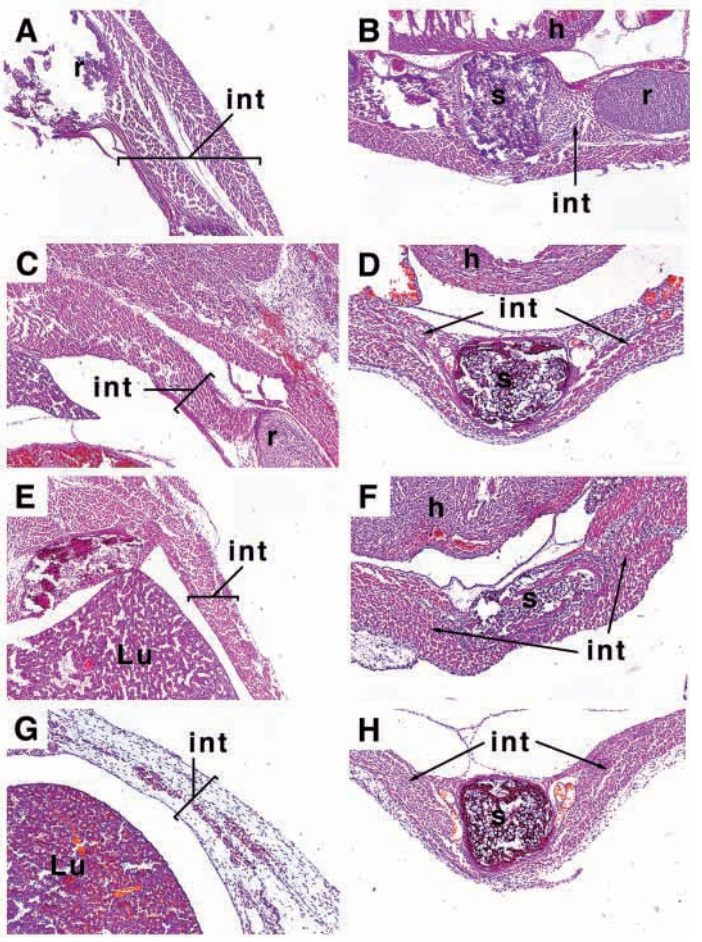

Fig. 8. *Myf5*$^{-/-}$/*paraxis*$^{-/-}$ neonatal mice develop distal but not proximal muscles of the body wall. Transverse thin sections were cut through the thoracic cavity and lower abdomen of wild-type (A,B), *paraxis*$^{-/-}$ (C,D), *myf5*$^{-/-}$(E,F), and *myf5*$^{-/-}$/*paraxis*$^{-/-}$ (G,H) neonates and stained with Hematoxylin and Eosin. Intercostal muscle proximal to the vertebrae were reduced to a single discontinuous layer of muscle in the absence of both Paraxis and Myf5. Intercostal muscles at the ventral midline are present at comparable levels in all three mutant backgrounds. H, heart; int, intercostals; Lu, lungs; r, ribs; s, sternum.

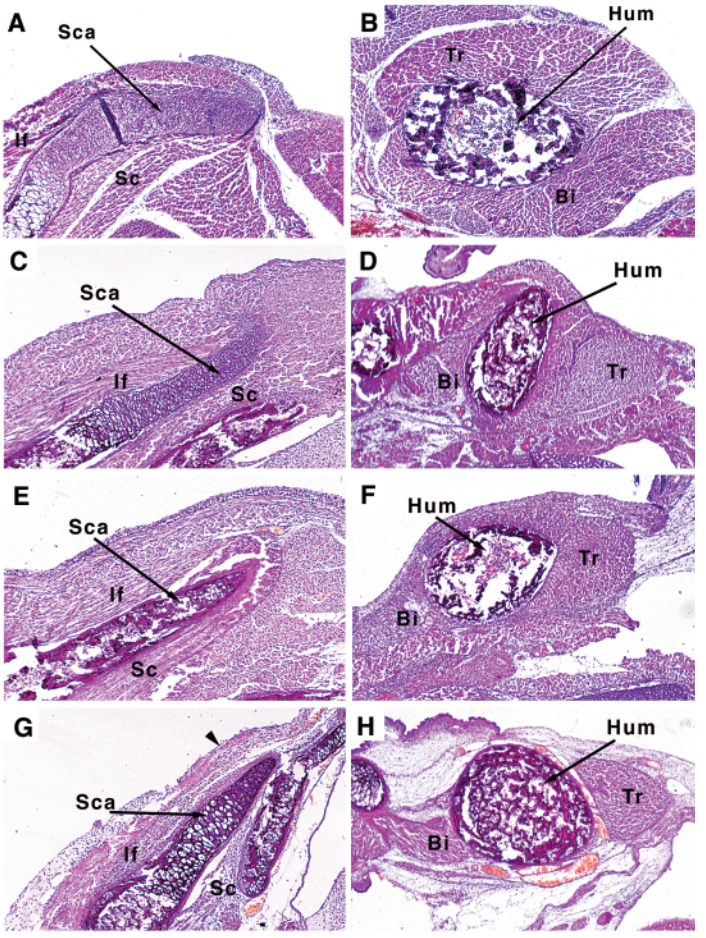

Fig. 9. *Myf5*$^{-/-}$/*paraxis*$^{-/-}$ neonatal mice display reduction of appendicular muscle. Transverse thin sections were cut through the forelimb level of wild-type (A,B), *paraxis*$^{-/-}$ (C,D), *myf5*$^{-/-}$ (E,F) and *myf5*$^{-/-}$/*paraxis*$^{-/-}$ (G,H) neonates and stained with Hematoxylin and Eosin. The supraspinatus and infraspinatus muscles are reduced and the subscapularis is absent in the *myf5*$^{-/-}$/*paraxis*$^{-/-}$ (G) but normal in the *paraxis*$^{-/-}$ (C) and *myf5*$^{-/-}$ (E) littermates. The triceps and biceps muscles are comparable in the *paraxis*$^{-/-}$ (D) and *myf5*$^{-/-}$ (F) and reduced in the *myf5*$^{-/-}$/*paraxis*$^{-/-}$ (H) neonates. Bi, biceps branchii; Hum, humerus; If, infraspinatus; Sc, subscapularis; Sca, scapula; Tr, triceps branchii

thought to be derived from the ventral edge of the myotome, and as such have been classified as non-migratory muscles.

Cell proliferation is altered in the dermomyotome of the *paraxis*-null embryos

It has been proposed that cell proliferation in the dermomyotome is important for the growth of the myotome. The propagation of a myogenic progenitor cell population that can be recruited to the myotome, beyond the initial migratory events, can be used to explain how the compartment continues to expand even though the myoblasts themselves are postmitotic (Christ and Ordahl, 1995; Kahane et al., 1998). We were interested in determining whether Paraxis is required for inducing and/or maintaining the proliferation state of the dermomyotome. Pregnant females from a *paraxis*$^{+/-}$ cross were injected with a BrdU cocktail intraperitoneally and then killed after 30 minutes. BrdU is a thymidine analog that will cross the placental barrier and rapidly become incorporated into newly synthesized DNA. Therefore, the presence of BrdU in the nucleus indicates that the cell has recently divided. In wild-type embryos at E9.5, all of the cells of the dermomyotome in both the thoracic and caudal somites were BrdU immunopositive (Fig. 10). In contrast, there were significantly fewer BrdU-positive cells in the dermomyotome of the *paraxis*$^{-/-}$ embryos. Within the dermomyotome of the mutants, the proliferating cells were consistently localized to the dorsomedial regions. This result raises the possibility that defects in the hypaxial myotome associated with the *paraxis*-null mutation may be due in part to the failure of the myogenic progenitor cells to proliferate at the dorsolateral lip of the dermomyotome.

DISCUSSION

The dermomyotome of the somite plays an important role in the genesis of skeletal muscle by acting as the primary source of cells for the initial formation of the myotome and its subsequent expansion. Whether these cells become fated to the epaxial or hypaxial muscles of the body is determined by instructive signals provided by the adjacent axial tissues (Aoyoma and Asamoto, 1988; Christ et al., 1992; Ordahl and Le Douarin, 1992; Marcelle et al., 1997). However, the genetic

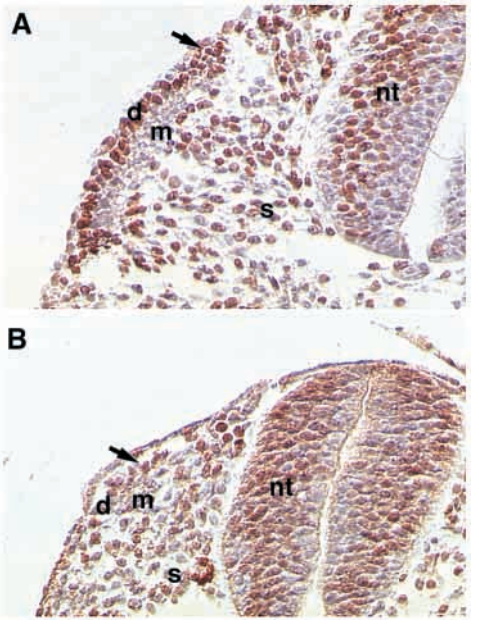

Fig. 10. Cells of the dermomyotome proliferate at a reduced rate in the absence of Paraxis. Mice at embryonic day 9.5 were exposed to BrdU for 30 minutes prior to being killed. Thin sections of either *paraxis*$^{+/+}$ (A) or *paraxis*$^{-/-}$ (B) embryos were immunostained for the incorporation of BrdU in the nucleus of dividing cells in the dermomyotome. The arrow denotes the dorsomedial edge of the dermomytome. d, dermomyotome; m, myotome; nt, neural tube; s, sclerotome.

pathway(s) within the myogenic progenitor cells that interpret these signals are poorly understood. Here, we demonstrate that Paraxis plays different roles in the formation of the epaxial and hypaxial myogenic lineages. In the medial myotome of *paraxis*$^{-/-}$ embryos, the myoblasts were disorganized and failed to form a parallel array of myocytes, and myoblasts did not appear at the lateral edge of the myotome until after E10.5. In contrast, cells from the dorsolateral lip of the dermomyotome failed to migrate into the hypaxial myotome in the absence of Paraxis. These observations point to Paraxis as an integral regulator of the myogenic lineages that give rise to the body wall muscle.

Formation of the medial myotome in the absence of the dorsomedial lip

The phenotype of the *paraxis*-null embryos at E9.5 raises an important question about the role of the dorsomedial lip in defining the organization of the epaxial myotome. It has been demonstrated in chick that the myogenic progenitors of the epaxial muscles are restricted to the rostromedial and medial edge of the dorsomedial lip (Christ and Ordahl, 1992; Denetclaw et al., 1997). These cells then migrate subjacently to populate the medial edge of the myotome. In this study, we used expression of a *myogenin-lacZ* transgene to analyze early events in epaxial myotome formation in the mouse. Migration of myoblasts and myocyte elongation as determined by transgene expression in wild-type embryos was comparable to the pattern described for the formation of the chick myotome (Denetclaw et al., 1997). Therefore, we can assume that expression of the *myogenin-lacZ* transgene in the *paraxis* mutants accurately depicts migration and differentiation of myoblasts.

Initially, we were interested in determining whether Paraxis was required for establishing the time at which myogenic progenitor cells enter the myotome. The tight link between the transition of cells from epithelium to mesenchyme and specification to the myogenic lineage would predict that the former may influence the latter. However, based on the expression of the *myogenin-lacZ* transgene, we concluded that cells were committed to the myogenic lineage at the same rate in the *paraxis* mutants as in wild-type littermates. This suggests that the genetic pathway that directs formation of the epaxial myotome from the dorsomedial lip of the dermomyotome functions independently of the epithelial state.

Our results indicate that Paraxis is required for the appropriate migration of myoblasts soon after entering the epaxial myotome. The signals that mediate the entry of myoblasts into the epaxial myotome and their ventral migration have not been established. The subsequent migration of myoblasts to peripheral sites, where they will differentiate to form muscle, is dependent on cytokines, cell adhesion and components of the extracellular matrix (ECM). It is likely that the same, or similar, mechanisms regulate early events in myotome formation. Candidate molecules for regulating these events include $\alpha 7\beta 1$ integrin, which mediates cell adhesion and migration of myoblasts on laminin (Yao et al., 1996; Crawley et al., 1997). Alternatively, counteradhesives such as SC-1, which is expressed selectively in the newly formed epaxial myotome, could stimulate migration by breaking interactions with the ECM (Ringuette et al., 1998). Paraxis could be regulating cell migration by controlling the transcription of any or all of the cell adhesion molecules involved. Alternatively, the basal lamina on the inner surface of the dermomyotome is disrupted in the *paraxis* mutants as a result of the failure of the somitic cells to form an epithelium. An analysis of laminin expression in the mutant embryos indicates that the transcription level within the somite was not altered but that the organization of the protein was disrupted (data not shown). This could also explain the aberrant organization of the myocytes as they elongate. It is possible that physical constraints on the path of migration into the myotome (Denetclaw et al., 1997) may be absent in the mutants. In the absence of an epithelial sheet with a basal lamina to act as a barrier, dermomyotomal cells may enter the myotomal compartment directly without first migrating to the medial edge. Further experiments will be required to resolve the nature of the regulation of myoblast migration by Paraxis.

Paraxis is required for the development of hypaxial muscles

The development of the hypaxial musculature from the dorsolateral lip of the dermomyotome occurs by two distinct mechanisms. In somites of the abdominal and thoracic regions, myoblasts migrate from the dorsolateral lip into the ventrolateral edge of the myotome. The dorsolateral lip is maintained in an epithelial state as the myotome expands into the lateral mesoderm, providing a continuous source of myoblasts that will give rise to the body wall musculature (Christ et al., 1983; Denetclaw et al., 1997). At the occipital and limb levels, the epithelial sheet of the dorsolateral lip delaminates with the individual cells dispersing to the periphery to eventually give rise to the limb muscles, the intrinsic muscles of the tongue, the diaphragm, and the pectoral and pelvic girdles (Jacob et al., 1978, 1979; Noden, 1983; Ordahl and Le Douarin, 1992).

Our analysis has identified Paraxis as an important regulator of the hypaxial muscles derived from the ventrolateral myotome based on several lines of evidence. First, the *myogenin-lacZ* transgene, which marks cells committed to the myogenic lineage, failed to be expressed in the ventrolateral region of the myotome at E10.5 in the *paraxis* mutants. Secondly, MyoD, which is required for the commitment of myoblasts in the ventrolateral myotome (Rudnicki et al., 1993; Kablar et al., 1999), is absent in this region of the myotome in the *paraxis* mutants at E10.5. Finally, mice deficient in both Paraxis and Myf5 suffered severe defects in the erector spinae, transversospinalis group and interspinalis muscles of the back (epaxials), and the abdominal and intercostal muscles (hypaxial) proximal to the spinal column. Muscles derived from the migratory cells of the dermomyotome were either unaffected (diaphragm and tongue) or mildly affected (appendicular muscle). A comparison of neonatal muscle development in the *paraxis*$^{-/-}$/*myf5*$^{-/-}$ mice to the reported phenotypes of the *myf5*$^{-/-}$/*MyoD*$^{-/-}$ and *myf5*$^{-/-}$/*Pax3*$^{Spl/Spl}$ mice revealed a distinct difference in muscle deficiencies (Rudnicki et al., 1993; Tajbakhsh et al., 1997). In both the *myf5*$^{-/-}$/*MyoD*$^{-/-}$ and *myf5*$^{-/-}$/*Pax3*$^{Spl/Spl}$ genetic backgrounds, the hypaxial muscles derived from the migratory cells of the dermomyotome are also absent. This has led us to the conclusion that Paraxis plays an important role in defining the non-migratory myotomal myoblasts of the ventrolateral myotome.

The presence of the muscles along the ventral midline of the

body wall in the *paraxis*$^{-/-}$/*myf5*$^{-/-}$neonates suggests that these muscles are regulated in a manner distinct from the remainder of the non-migratory hypaxial muscles that populate the trunk. These muscles are deficient in *Splotch* mice, suggesting that they are dependent on the expression of Pax3 (Tremblay et al., 1998). However, c-Met, which mediates somitic cell migration is not detectable in ventral myotome nor is the formation of the rectus abdominus and distal intercostal muscles affected in *c-met*$^{-/-}$ mice (Bladt et al., 1995). This suggests that they are not regulated by the same mechanism as the migratory hypaxial muscles. Therefore, we propose that these muscles fall into a new class of hypaxial muscles that are maintained in a proliferative state at the ventral edge of the expanding myotome and further that these muscles are independent of Paraxis.

Paraxis regulates the transcription of *Pax3* in the dermomyotome

Expression of *Pax3* in the dermomyotome has been demonstrated to be essential for both the formation of the hypaxial myotome and the migration of the myogenic progenitor cells into the limb (Franz et al., 1993; Goulding et al., 1993; Tajbakhsh et al., 1997; Tremblay et al., 1998). It appears to function as a mitogen, thus allowing for the expansion of the myogenic progenitor cells in the dorsolateral dermomyotome and the migratory populations of the limb (Amthor et al., 1998, 1999). By maintaining these cells in a proliferative state, *MyoD* transcription is blocked and progression through muscle differentiation is blocked. Pax3 appears to regulate cell migration by controlling the transcription of the tyrosine kinase receptor, *c-met* in the cells of the lateral dermomyotome (Yang et al., 1996). Interaction between c-Met and its ligand hepatocyte growth factor (HGF) in turn mediates the migration of somitic cells into the periphery.

There are strong similarities in the defects in myotome formation between the *paraxis* and *Pax3* mutant embryos at E9.5 and E10.5. The organization of the myocytes in the medial myotome is disrupted, the formation of lateral myotome and its subsequent ventral migration are delayed (Tremblay et al., 1998). This suggests that *paraxis* and *Pax3* lie in a common genetic pathway controlling the formation of the myotome from myogenic precursors in the dermomyotome. An analysis of *Pax3* transcription in the *paraxis*$^{-/-}$ embryos, revealed a complex pattern of gene regulation, further strengthening this conclusion. At E9.5, *Pax3* was expressed in the segmental plate and newly formed somites but was lost at the time of compartmentalization, suggesting that Paraxis is required to maintain transcription. The following day Pax3 was detectable in the ventrolateralmost aspects of the dermomyotome in the trunk and the muscle primordia of the limbs. *Pax3*-expressing cells in the migratory hypaxial muscle precursor cells are independent of *paraxis* expression, which would be consistent with the presence of these muscles in the *myf5*$^{-/-}$/*paraxis*$^{-/-}$ neonates.

Paraxis is required for proliferation of the dorsolateral dermomyotome

Growth of the myotome is dependent on the continued migration of cells from a proliferative myogenic progenitor cell population in the dorsolateral lip of the dermomyotome. Upon entering the myotome these cells become postmitotic (Kaehn et al., 1988). We observed a reduction in the proliferation of cells in the dorsolateral half of the dermomyotome in E9.5 *paraxis*$^{-/-}$ embryos as measured by incorporation of the thymidine analog, BrdU. It is possible in these embryos that migration of cells into the myotome and limb would rapidly deplete myogenic progenitor population, effectively blocking subsequent growth of the lateral myotome. Cells that enter the limb field continue to proliferate, presumably due to the expression of Pax3 (Amthor et al., 1998, 1999), thus allowing this population to overcome any initial reduction in progenitors. It remains to be determined if Paraxis is directly responsible for maintaining proliferation of cells in the dermomyotome. Since Pax3 is able to regulate cell proliferation, it is possible that Paraxis modulates proliferation through controlling *Pax3* transcription. The decision of a cell to proliferate or exit the cell cycle and differentiate is a key developmental event during myogenesis. Further studies will be required to delineate the role of Paraxis and Pax3 in this process.

Paraxis acts as an intermediate in the induction of hypaxial myogenesis by the surface ectoderm

Elegant studies in chick have demonstrated that somites are naïve tissue at the time of formation that are patterned by instructive signals from the surrounding axial structures (Aoyoma and Asamoto, 1988; Christ et al., 1992; Ordahl and Le Douarin, 1992; Marcelle et al., 1997). These signals impose a complex spatial and temporal regulation upon the specification, differentiation and migration of myogenic progenitors. Specification of the hypaxial musculature in the dorsolateral lip of the dermomyotome occurs in response to a combination of dorsalizing signals from the surface ectoderm and lateralizing signals from the lateral mesoderm (Fan and Tessier-Lavigne, 1994; Pourquié et al., 1995; Cossu et al., 1996; Dietrich et al., 1998; Capdevila and Johnson, 1998). Candidate signals from the surface ectoderm include members of the Wnt family (Wnt1, Wnt3a, Wnt4, Wnt6 and Wnt7a), which are able to induce the transcription of Pax3 and the myogenic factors in segmental plate explant co-culture experiments (Munsterberg et al., 1995; Fan et al., 1997; Maroto et al., 1997; Dietrich et al., 1998; Tajbakhsh et al., 1998). Since these signals act at short range or by cell-cell contact, it is likely that the inductive target of the surface ectoderm is the myogenic progenitor cells in the dermomyotome (Fan and Tessier-Lavigne, 1994). The lateral mesoderm, in turn expresses BMP4, which is able to synergize with dorsalizing signals to increase Pax3 transcription in the a dose-dependent manner (Pourquie et al., 1996; Dietrich et al., 1998; Amthor et al., 1999).

It remains to be determined how these extrinsic signals are differentially interpreted by the migratory and non-migratory myogenic progenitors of the dorsolateral dermomyotome. It appears that specification of the migratory cells require signals from the lateral mesoderm. Evidence of this is the expression of migratory cell-specific gene, *Lbx1*, in paraxial mesoderm in the presence of lateral plate mesoderm or BMP4 (Dietrich et al., 1998). In addition, hepatocyte growth factor (HGF), expressed in the lateral mesoderm is required for epithelial breakdown of the dorsolateral lip of the dermomyotome and migration of cells into the limb (Brand-Saberi et al., 1996; Heymann et al., 1996; Dietrich et al., 1999). These observations demonstrate the importance of the lateral mesoderm in establishing migratory myogenic cells. Here we demonstrate that *paraxis* expression is

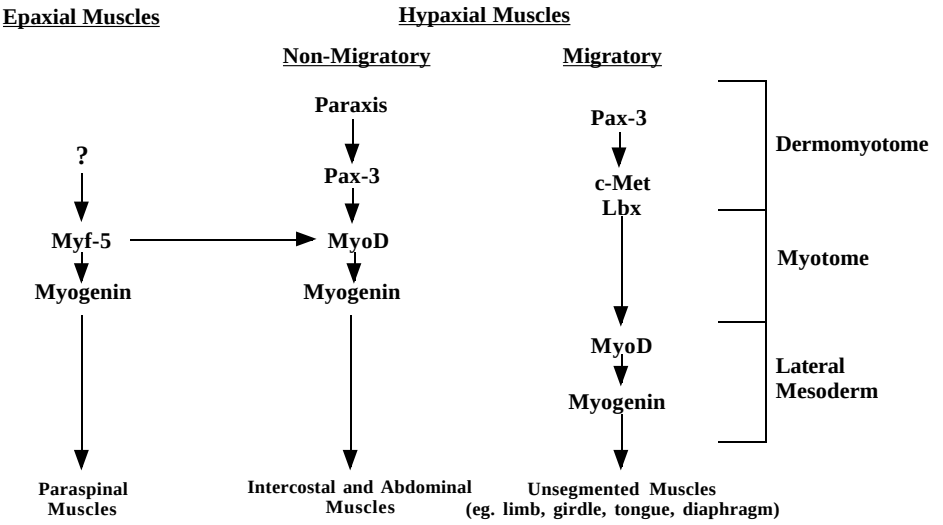

Fig. 11. A model of the regulatory cascade leading to the formation of somitic-derived skeletal muscle. Arrows do not necessarily indicate direct regulation of transcription.

required for the formation of the non-migratory hypaxial muscle. Since *paraxis* transcription is induced by signals from the surface ectoderm (Sosic et al., 1997), it is likely that the expression of this gene in dermomyotomal cells is an intermediate step in the induction of myogenesis. The fact that *paraxis* transcription is not induced by lateral mesoderm suggests that the non-migratory hypaxial muscles are not responsive to signals from the lateral mesoderm. Consistent with these observations, one of the functions of Paraxis, maintenance of the dermomyotome in an epithelial state, is counteracted by HGF. It will be important to determine the relationship between the functions of Paraxis and HGF.

Expression of members of the bHLH transcription factor superfamily in paraxial mesoderm have been demonstrated to regulate morphogenesis, determination and differentiation. In a previous report, it was established that Paraxis functions as a morphoregulator, controlling the epithelial state of the somite. The data presented here has led us to conclude that Paraxis is also required to specify myogenic precursors in the dermomyotome. We propose a model of two regulatory pathways, leading to the formation of hypaxial muscle, that are distinguished by their requirement for Paraxis (Fig. 11). Non-migratory myoblasts, which give rise to the proximal intercostal and abdominal wall muscles, are initially activated by the expression of *myf5* in the myotome (Tajbakhsh et al., 1997). Continued growth of the dorsolateral myotome then becomes dependent on *paraxis*-expressing cells in the dorsolateral dermomyotome. Paraxis is required for expansion of this precursor cell population and the maintenance of *Pax3* transcription. Alternatively, myogenic precursor cells fated to migrate into the periphery, are able to express *Pax3* independent of *paraxis*. A complete understanding of the mechanism by which Paraxis regulates myogenesis awaits the identification of downstream target genes.

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