Regulation of Pax-3 expression in the dermomyotome and its role in muscle development

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SUMMARY

The segmented mesoderm in vertebrates gives rise to a variety of cell types in the embryo including the axial skeleton and muscle. A number of transcription factors containing a paired domain (Pax proteins) are expressed in the segmented mesoderm during embryogenesis. These include Pax-3 and a closely related gene, Pax-7, both of which are expressed in the segmental plate and in the dermomyotome. In this paper, we show that signals from the notochord pattern the expression of Pax-3, Pax-7 and Pax-9 in somites and the subsequent differentiation of cell types that arise from the somitic mesoderm. We directly assess the role of the Pax-3 gene in the differentiation of cell types derived from the dermomyotome by analyzing the development of muscle in splotch mouse embryos which lack a functional Pax-3 gene. A population of Pax-3-expressing cells derived from the dermomyotome that normally migrate into the limb are absent in homozygous splotch embryos and, as a result, limb muscles are lost. No abnormalities were detected in the trunk musculature of splotch embryos indicating that Pax-3 is necessary for the development of the limb but not trunk muscle.

Key words: Pax-3, somites, notochord, muscle differentiation, splotch mutation, myoD

INTRODUCTION

The patterning and differentiation of cells in the somitic mesoderm is an important step in the development of axial structures that include the vertebral column and skeletal muscle. Development of the somitic mesoderm is characterized by the progressive segmentation of mesoderm tissue directly flanking the neural tube into two bilateral rows of somites. Somites when they first appear consist of radially arranged epithelial balls of mesoderm called epithelial somites. These epithelial somites then undergo a number of morphological changes that eventually result in their dissolution and differentiation into a variety of tissues including bone, cartilage, dermis and muscle (reviewed in Keynes and Stern, 1988). An early morphological change during somite maturation occurs when cells in the ventromedial part of the somite lose their epithelial arrangement and form a loosely arranged mesenchyme from which sclerotome cells are derived. At the same time, cells in the dorsal part of the somite retain their epithelial organization and form the dermomyotome. The dermomyotome subsequently develops into two structures, an outer dermatome cell layer and an inner myotome cell layer. Both the axial and appendicular muscles and dermis are generated from the dermomyotome. In the chick embryo, two populations of myogenic precursors have been identified in somites. The striated muscles of the axial musculature derived from the medial part of the somite and limb muscles that develop from the lateral somite (Ordahl and Le Douarin, 1992). Initially both the lateral and medial somite halves are capable of generating limb or trunk muscles; however, this is lost as the somites mature (Aoyama and Asamoto, 1988; Ordahl and Le Douarin, 1992). Muscle precursor cells for the limb musculature are derived from the lateral portion of the dermomyotome adjacent to the limb bud. These cells migrate into the limb bud where they differentiate and fuse to form the primary myotubes of the limb muscles (Chevallier et al., 1977; Christ et al., 1977).

Although the events governing the development of the somite and the generation of different cell types within the somite are poorly understood, embryonic manipulations in the chick embryo have provided insights into the nature of the interactions that regulate somite development. Experiments manipulating somites in the chick embryo are consistent with cell fate in the early somite being determined by the interaction of somitic cells with adjacent tissues (Aoyama and Asamoto, 1988; Ordahl and Le Douarin, 1992). Muscle precursor cells for the limb musculature are derived from the lateral portion of the dermomyotome adjacent to the limb bud. These cells migrate into the limb bud where they differentiate and fuse to form the primary myotubes of the limb muscles (Chevallier et al., 1977; Christ et al., 1977).

For example, implanting an extra notochord adjacent to somites causes the loss of the dermomyotome from regions adjacent to the graft and the development of ectopic vertebrae (Pourquie et al., 1993). In contrast, removing both the notochord and neural tube from E2 chick embryos results in the loss of both sclerotome and myotome (Rong et al., 1992), suggesting that...
the notochord/neural tube complex is required for the survival and development of both tissues. The neural tube and skin epithelium may also provide signals that are necessary for the development of the myotome, since both increase myogenesis in isolated somites when co-cultured with them (Kenny-Moobbs and Thorogood, 1987). Taken together these results indicate that tissues adjacent to the somite, including the neural tube and the notochord, are able to regulate the differentiation of cell types derived from the somite. These results parallel the demonstrated role of the notochord and floor plate in establishing dorsoventral polarity and cell identity in the spinal cord (Placzek et al., 1990; Yamada et al., 1991).

The Pax genes are a family of paired box-containing genes that encode for cell-specific transcription factors. All of the Pax genes are expressed in discrete regions of the developing embryo, where they are postulated to regulate pattern formation. Four mouse Pax genes and their chick homologues are expressed in the somitic mesoderm and cells derived from the somitic mesoderm. Pax-3 and Pax-7 are expressed in the paraxial mesoderm prior to segmentation. In somites, transcripts of Pax-3 and Pax-7 are restricted to the prospective dermomyotome and later to the myotomes and to myoblast cells in the limb that are derived from the dermomyotome (Goulding et al., 1991; Jostes et al., 1990). Two other genes, Pax-1 and Pax-9, are expressed in the sclerotome (Deutsch et al., 1988; R. Balling, personal communication). Pax-1 is expressed in the ventral somite in mesenchymal cells lateral to the notochord of embryonic day 10 mouse embryos. In day 12 embryos, Pax-1 is expressed in perichondral condensations around the notochord and subsequently in the intervertebral discs (Deutsch et al., 1988). Pax-9, a gene closely related to Pax-1, is expressed in somites beginning around stage 12 in the chick, in the sclerotome and later in axial cartilage (M. G., unpublished data).

While the restricted expression of the Pax genes in developing somites suggests a role in cell patterning, the exact function of the Pax genes in somite morphogenesis is unknown. Evidence for the involvement of the Pax genes in somite development has come from the mouse mutant undulated (Gruneberg, 1954). A point mutation in the paired box of Pax-1 gene that severely reduces DNA binding by the Pax-1 protein is responsible for the axial skeleton defects in undulated mice (Balling et al., 1988; Chalepakis et al., 1991). The role of the other Pax genes in the morphogenesis of somites remains unclear. As a first step toward determining the role, if any, that Pax-3 plays in the differentiation of somites, we have examined the effect of the notochord on the expression of Pax-3 in somites and on the differentiation of somitic cells. Previously, we have shown that signals from the notochord regulate the regional expression of Pax-3 and Pax-6 in the spinal cord of chick embryos (Goulding et al., 1993). In these experiments, a close correlation was observed between the early changes in Pax gene expression and the previously described patterning of cell types in the spinal cord by the notochord (van Straaten et al., 1988; Placzek et al., 1990; Yamada et al., 1991; van Straaten and Hekking, 1991; Ericson et al., 1992). In this paper, we show that the notochord regulates Pax gene expression and cell differentiation in the somitic mesoderm and that these changes closely parallel the changes to Pax gene expression that occur in the adjacent spinal cord. In addition, we provide evidence that Pax-3 plays a role in the development of a subset of muscles derived from the somitic mesoderm. Pax-3-expressing myogenic precursor cells are absent from the limbs of homozygous splotch embryos and, as a result, these mice fail to develop limb muscles.

MATERIALS AND METHODS

Embryos

White Leghorn hens’ eggs were incubated at 37.6°C in a humidified forced-draft incubator. Eggs were windowed after 2 days and staged according to Hamburger and Hamilton, 1951. Following the operation, eggs were sealed and incubated for a further 8–96 hours at 37.6°C.

Mice used in this study were obtained from the Jackson Laboratory, Bar Harbor, Maine and from the MRC Radiobiology Laboratory, Harwell, UK. Mouse embryos were obtained from timed matings of C57BL/6, Sp, Sp1H, Sp2H mice. The morning of the plug was designated day 0.5. Homozygous splotch embryos were identified by the presence of spina bifida and exencephaly.

Notochord removal

Notochords were surgically removed from stage 10 embryos in ovo. White Leghorn hens' eggs were incubated to Hamburger-Hamilton stage 10 at 37.6°C. Eggs were windowed, India ink injected beneath the blastoderm and a small region of the vitelline membrane removed. The posterior neural plate was reflected by cutting through the ectoderm around its perimeter. The terminal 200-300 µm of the notochord was excised and the neural plate returned to its original position. Embryos (n=9) were then incubated for a further 2 days at 90% relative humidity. Embryos were removed on day 4, fixed and embedded in paraffin. Notochord ablations (n=12) were also performed on stage 9-10 chick embryos grown in vitro from stage 4-5 according to the method of New (1955). Embryos were allowed to develop until H-H stages 14-16 before analyzing Pax-3 and myoD expression by whole-mount in situ hybridization.

Notochord implantation

Notochords were implanted at specific lateral positions beside the segmental plate of stage 10-11 embryos. A longitudinal incision was made directly beside the open caudal neural plate at the level of presumptive somite numbers 10-17. Donor notochord pieces (300-400 µm in length) from stage 10-12 embryos were inserted into the opening and manipulated into position at the required lateromedial (ultimately dorsoventral) position next to the neural plate. Eggs were ressealed and incubated at 90% RH for a further 2-3 days. Surviving notochord-implanted embryos (n=37) were fixed and examined at stage 21-25 or, in some experiments, 8-16 hours after operating. Transverse sections from embryos spanning the notochord implants were examined by in situ hybridization for expression of Pax-3, Pax-7, Pax-9 and myoD. Sections were stained with Giemsa and cover slipped, and then examined and photographed under bright-field/dark-field illumination for gene expression and histology.

In situ hybridization

Embryos were removed and fixed in 3.5% formaldehyde in 50 mM phosphate buffer pH 7.2. Following fixation, embryos were processed either for paraffin or cryostat sections. Transverse sections (7-8 µm) were collected on gelatin-subbed slides and pretreated for in situ hybridization. A 182 bp fragment encompassing the homeobox and octapeptide sequences was used for Pax-3 and a 582 bp fragment including part of paired and homeobox was used for Pax-7. For in situ studies with Pax-9, a 188 bp probe from the paired box region of Pax-9 was used and for chicken myoD, a 476 bp fragment from the 3’ untranslated region of the chicken myoD gene, CMD1 (Lin...
et al., 1989), was used. In situ hybridization was performed as described in Goulding et al. (1993). Sections were photographed using a Nikon Optiphot microscope with dark-field/bright-field illumination.

**Whole-mount in situ hybridization**

For whole-mount in situ hybridization, embryos were fixed overnight in 3.5% paraformaldehyde, washed twice in PBT (PBS, 1% Tween-20), dehydrated through 25%, 50%, 75% and 100% methanol, and stored at −20°C. Following rehydration, whole-mount in situ hybridization was performed as described in Goulding et al. (1993). For photography, embryos were either mounted whole in 80% glycerol/PBS or embedded in agar-sucrose and vibratome sectioned at 60-70 µm. Sections, mounted on subbed slides, were photographed using a Nikon Optiphot microscope with bright-field illumination.

**Acetylcholinesterase assay**

Vibratome sections (100 µm) from wild-type and homozygous splotch embryos were collected on gelatin-subbed slides and air dried for 10 minutes. Acetylcholinesterase activity was detected using the method of Koelle and Friedenwald (1949). Sections were coverslipped and photographed as described above.

**RESULTS**

**Expression of Pax-3 in segmented mesoderm**

The Pax-3 gene encodes a putative transcription factor containing two conserved DNA-binding domains, the paired domain and the homeodomain. Previously, we have shown the Pax-3 protein is highly conserved in vertebrates, with the chicken and mouse proteins sharing 97% identity at the amino acid level (Goulding et al., 1993). The high degree of protein conservation, together with the near identical patterns of Pax-3 expression in the mouse and chick indicate the function of Pax-3 has been conserved in widely divergent vertebrate species. In the mouse, Pax-3 is expressed in the developing nervous system and in the somitic mesoderm beginning at day 8 of gestation and in the limb buds of day 10.5 embryos (Goulding et al., 1991). In order to understand better the role Pax-3 plays in the development of the somitic mesoderm and cell types derived from somites, we have undertaken a detailed analysis of the expression of Pax-3 in the chick embryo. We describe the expression of the chicken Pax-3 gene in the develop
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oping somitic mesoderm and analyze the effect of the notochord on Pax-3 expression in somites. Expression of Pax-3 is first observed in the somitic mesoderm of chick embryos at stage 7. By stage 8, Pax-3 expression can be seen in both the segmental plate and in newly formed somites (Goulding et al., 1993). In the chick embryo, somites are progressively generated in a rostrocaudal direction, such that rostral somites are more mature than caudal somites. In order to follow the developing expression pattern of Pax-3 in somites, we have examined the expression of Pax-3 at the level of the segmental plate, the most recently formed somite and in cervical somites from stage 13-15 chick embryos. These results are shown in Fig. 1. Initially, Pax-3 is expressed in cells throughout the segmental plate and is not dorsoventrally restricted in its expression pattern (Fig. 1B). As the epithelial somites separate from the segmental plate, Pax-3 expression is restricted to cells in the dorsal somite, the presumptive dermatomyotome (Fig. 1C). This occurs before cells in the ventral somite undergo the epithelial-to-mesenchymal transition that marks the emergence of sclerotome. In more mature somites at the cervical level, Pax-3 expression is restricted to the dermatomyotome and is absent from the myotome (Fig. 1D).

At later stages of somite morphogenesis, a further change in the expression pattern of Pax-3 in somites occurs. In stage 18 embryos, Pax-3 is expressed at a higher level in the lateral dermatomyotome than in the medial part of the dermatomyotome (Fig. 2A,C). The myogenic precursors of the limb muscles are believed to arise from the lateral part of the dermatomyotome. These cells express Pax-7 at a lower level than cells in the medial part of the dermatomyotome (Fig. 2B,D). Prior to this Pax-7 is expressed throughout the dermatomyotome indicating Pax-7 is down regulated in these cells just before limb muscle precursors begin to leave the somite. These differences in the expression patterns of Pax-3 and Pax-7 in somites were seen in both the mouse and the chick.

**Pax-3 expression in somites is repressed by an ectopic notochord**

Previously we have shown that signals from the notochord and from the floor plate are able to regulate the expression of Pax-3...
3 in the spinal cord (Goulding et al., 1993). Given the parallels between the dorsally restricted expression of Pax-3 in the spinal cord and somites, we analyzed the effect of the notochord on the expression of Pax-3 in somites. Pieces of notochord were grafted into stage 10-11 embryos adjacent to the segmental plate and the posterior-most somite. Expression of Pax-3 was then analyzed 8 and 16 hours after the operation. When a second notochord was placed in a dorsal position adjacent to the somitic mesoderm, a dramatic change in the expression of Pax-3 in newly formed somites was seen. Normally Pax-3 is expressed in the dorsal part of the somite from the earliest stages of somite development. In embryos with an ectopic notochord, Pax-3 was down-regulated in the segmental plate and in somites immediately adjacent to the notochord graft (Fig. 3) at a time when the most recently formed somites still consist of an epithelial sphere of cells that have yet to differentiate into dermomyotome or sclerotome (Fig. 3B).

When embryos were examined at 16 hours after the operation, a clear difference in the morphology of cells adjacent to the ectopic notochord was seen. Cells in the dorsal part of the somite immediately adjacent to the notochord graft (n”) lacked the normal epithelial organization characteristic for this stage of somite development, while somites on the contralateral side of the graft appeared normal in both morphology and expression of Pax-3 in the dermomyotome (Fig. 4A,B). Cells in the dorsal somite adjacent to the notochord did not express Pax-3 and were morphologically indistinguishable from the ventral sclerotome cells. The change in the morphology of these cells in response to the notochord graft closely resembles the change in the morphology of ventral somite that normally occurs during sclerotome development. In sham-operated embryos, no such changes were observed (not shown), indicating that these changes to the dermomyotome do not result from trauma or surgery. Embryos examined 2 and 3 days after the grafting operation showed similar repression of Pax-3 in the mesoderm adjacent to the ectopic notochord (Fig. 5B,E). At these later stages of development, a difference in the morphology of the mesoderm in the vicinity of the ectopic notochord was noted (Fig. 5A,D). In stage 21 embryos, no myotome was visible on the side of the notochord graft and by stage 25, extra cartilage tissue was present around the ectopic notochord in regions where muscle tissue would normally develop (Fig. 5D,G).

**Signals from the notochord repress Pax-7 and myoD expression in somites**

To examine further the effect of the notochord on cell differentiation in the somite, the expression patterns of Pax-7, myoD and Pax-9 were examined in embryos containing a supernumerary notochord. Pax-7 is a paired box gene closely related to Pax-3, which is expressed in the dermomyotome and muscle precursors in the mouse (Jostes et al., 1990). myoD encodes a helix-loop-helix (HLH) transcription factor that was first identified by its ability to convert fibroblast cells into myoblasts that then fuse to form myotubes (Davis et al., 1987). In the avian embryo, myoD is specifically expressed in the myotome, with transcripts first appearing in the medial part of the myotome 3-4 somites anterior to the last somite to form (Pownall and Emerson, 1992). At later stages of development, myoD is expressed throughout the myotome and in limb myoblasts (Pownall and Emerson, 1992).

In embryos bearing an extra notochord, a dramatic change in the expression pattern of Pax-7 in the somitic mesoderm was seen (Fig. 5F). On the side of the embryo bearing the ectopic notochord, Pax-7 was no longer expressed in cells adjacent to the ectopic notochord, in regions that normally differentiate into myotome (Fig. 5D). Moreover, the notochord-induced change in Pax-7 expression in the mesoderm matched closely the alteration to Pax-3 expression seen in adjacent sections (Fig. 5E,F). In all embryos examined, the loss of Pax-3 and Pax-7 expression in the vicinity of the notochord graft was
accompanied by the loss of a morphologically discernible myotome, indicating that the presence of an extra notochord prevents mesodermal cells nearby from differentiating into myotome, causes their dispersal, or both.

The alterations to Pax-3 and Pax-7 expression in response to a supernumerary notochord suggested that muscle differentiation was being inhibited by the ectopic notochord. To examine the effect of the notochord on myotome differentiation further, the expression of the chicken myoD gene (CMD1) in somites was also analyzed. Transcripts of myoD were absent from regions of the mesoderm adjacent to the ectopic notochord that normally give rise to the myotome and muscle tissue (Fig. 5H). Histological examination of the mesoderm tissue around the ectopic notochord showed cartilage tissue in regions of the embryo where the myotome was no longer present (Fig. 5D,G). These results show a close correlation between myoD repression by the notochord and the down regulation of Pax-3 and Pax-7 expression. The fact that the notochord is able to rapidly down regulate Pax-3 (Fig. 3) and Pax-7 (not shown) in somites suggests that changes in the expression of both genes may be early events in the repression of muscle differentiation by the notochord.

**Pax-9 is expressed in cells adjacent to the notochord graft**

Recently, it has been reported that an ectopic notochord can induce sclerotome differentiation (Pourquie et al., 1993). *Pax-9* is expressed both in sclerotome cells and in perinotochordal cells in the developing vertebral column and therefore can serve as a marker for condensing sclerotome. In order to characterize the identity of mesoderm cells surrounding the ectopic notochord further, *Pax-9* expression was analyzed in sections adjacent to those used for *myoD* expression. When a second notochord was placed in the segmental plate of stage 10 embryos and embryos were examined at stages 21 and 25, an increase in *Pax-9* expression on the side of the embryo bearing the notochord graft was observed adjacent to the ectopic notochord where myotome would normally be present (Fig. 5I). These cells normally express Pax-3, Pax-7 and myoD. The observation that cells surrounding the notochord graft expressed *Pax-9* instead of the muscle-specific marker *myoD* further suggested they had differentiated into chondrocytes (c.f. Fig. 5H,I). The increase in *Pax-9*-expressing cells near the ectopic notochord was closely associated with the differentiation of cartilage tissue and the development of a second vertebral mass (see arrow in Fig. 5G). The presence of cartilage tissue around the notochord graft was confirmed by staining...
Regulation of Pax gene expression in somites and muscle.
adjacent sections with basic fuchsin-haematoxylin (not shown).

**Effect of notochord removal on Pax gene expression in somites**

The experiments examining the effects of an ectopic notochord on Pax gene expression and cell differentiation in the somitic mesoderm suggested that the notochord may provide signals necessary for sclerotome differentiation. To analyze this further, the effect of notochord removal on the expression of Pax-3, Pax-7, myoD and Pax-9 in the somitic mesoderm was examined. A 200-300 µm piece of notochord was removed from beneath the neural plate of stage 10 embryos and these were then allowed to develop to stages 15 and 21. In embryos lacking a notochord, the morphology of the somites was severely disrupted, leading in many cases to the fusion of somites beneath the spinal cord (Fig. 6A).

A dramatic change was seen in the expression of all four genes in embryos lacking a notochord. In stage 15 notochord-less embryos, Pax-3 was now expressed in cells lying directly beneath the neural tube where the sclerotome is normally located (Fig. 6A,B). Later stage embryos lacking a notochord also exhibited similar ventral shift in the expression domains of Pax-3 expression and Pax-7 (Fig. 6D,F). When myoD expression was examined in stage 15 embryos lacking a notochord, the distribution of myoD transcripts in the somitic mesoderm was also dramatically changed. myoD is normally expressed in the myotome in two bilateral rows of somites flanking the neural tube. In embryos lacking a notochord, myoD was expressed in fused somites only in cells positioned directly beneath and contacting the neural tube (see arrow in Fig. 6E). Expression of Pax-3 in these cells appeared to be down regulated. Interestingly, myoD expression was completely lost from somites positioned some distance from the residual notochord, suggesting that notochord signaling to the neural tube may be necessary for myoD induction (data not shown). In contrast to the changes in Pax-3, Pax-7 and myoD expression, Pax-9 transcripts were completely absent from the mesoderm in regions of the embryo missing the notochord (Fig. 6G,H). The loss of Pax-9 expression in embryos lacking a notochord, together with the changes in Pax-3, Pax-7 and myoD expression, suggest that signals from the notochord are necessary for sclerotome development and expression of Pax-9.

**Expression of Pax-3 in the limb**

In addition to expression in the somitic mesoderm, Pax-3 is expressed in the developing limbs (Goulding et al., 1991). The expression of Pax-3 in the developing limb was examined in detail by in situ hybridization to ascertain the identity of cells expressing Pax-3 in the limb. Cells expressing Pax-3 first appear in the limb bud field at stage 19. These cells are located close to the lateral edge of somites where the limbs will form (Fig. 7A) and appear to be migrating from the lateral edge of the dermomyotome into the presumptive limb bud. As the limb develops further, increasing numbers of Pax-3-expressing cells can be seen in the proximal limb bud where they are intermingled with mesenchymal cells (Fig. 7B-D). In transverse sections through the limb bud, two populations of Pax-3-expressing cells can be seen. From their position, these cells appear to be the precursors for the dorsal and ventral muscle masses (Fig. 7C,D). At later stages of limb development, Pax-3 and myoD are expressed together in limb muscles (Fig. 7E-G). However, it is not known if both genes are co-expressed in myoblasts or whether Pax-3-expressing cells are intermingled with differentiating myoblasts in the limb.

**Limb muscles are absent in Pax-3− (splotch) embryos**

The close correlation between the expression of Pax-3 in somites and the emergence of the dermomyotome suggested that Pax-3 might regulate the differentiation of the dermomyotome and/or cell types derived from the dermomyotome. In order to examine further the role of Pax-3 in the dermomyotome, the differentiation of skeletal muscle was examined in splotch embryos. A number of mutations in the Pax-3 gene have recently been identified in various alleles of splotch (Epstein et al., 1991, 1993; Goulding et al., 1993). Two alleles, Sp and Sp2H, are believed to be null alleles and contain mutations that severely disrupt the protein-coding region of the Pax-3 gene (Epstein et al., 1991; Goulding et al., 1993).

Muscle development in wild-type and homozygous splotch embryos was analyzed by examining the expression of the myogenic determination factor myogenin in embryonic day 12.5 wild-type and splotch embryos. Myogenin is one of four basic helix-loop-helix DNA-binding proteins that are expressed during myogenic differentiation (Olson, 1991). Inactivation of myogenin in mice leads to severe defects in skeletal muscles, demonstrating the importance of myogenin expression for skeletal myogenesis (Hasty et al., 1993). Expression of myogenin was examined by in situ hybridization using a 0.9 kb mouse myogenin probe containing sequences from the coding region of the myogenin gene (Yee and Rigby, 1993). In sections through the forelimb region of day 12.5 wild-type embryos, transcripts of myogenin were detected in axial muscles and in differentiating muscle cells located in the shoulder and proximal part of the developing limb (Fig. 8A,B). In contrast to wild-type embryos, no expression of myogenin was detected in the forelimbs of day 12.5 Sp (Fig. 8C,D) and Sp2H embryos (Fig. 8E,F). Moreover, myogenin was still
expressed in the trunk region of splotch embryos, in both the intercostal and axial musculature (Fig. 7). When expression of myogenin and myf-5 was examined in northern blots of total RNA from day 12 wild-type and Sp embryos, only small differences were found in the relative levels of gene expression between wild-type and mutant embryos (not shown), reflecting the specific loss of limb muscles in splotch embryos.

The enzyme acetylcholinesterase is expressed in a range of cell types in the central and peripheral nervous system and in muscle tissue. To analyze further the effect that loss of Pax-3
function has on muscle development, the distribution of acetylcholinesterase was examined in tissue sections from wild-type and heterozygous splotch (Sp) embryos. In day 13 wild-type embryos, acetylcholinesterase activity was seen in the individual muscle masses of the upper forelimb and in the brachial nerve that innervates the forelimb (Fig. 8G). In splotch embryos, acetylcholinesterase activity was markedly depleted in the forelimb (Fig. 8H). No muscle-specific acetylcholinesterase staining was seen in the forelimbs and hindlimbs of splotch embryos. In marked contrast to the limbs, the distribution of acetylcholinesterase in the axial or body wall muscles of day 13 mutant embryos (not shown) did not differ significantly from wild-type embryos, indicating that the morphology of these muscles, as revealed by acetylcholinesterase activity, was normal.

In the mouse, migration of Pax-3-expressing cells into the limb field begins around day 10 in the forelimb (Fig. 7D). When Pax-3 expression was examined in day 10.5 homozygous splotch (Sp) embryos, no Pax-3-expressing cells were detected in the forelimb or hindlimb, suggesting that these cells had not migrated from the ventral somite (Fig. 9C,D). In wild-type littermate controls, Pax-3-expressing cells were present as expected in two populations of migrating cells in the dorsal and ventral limb bud (Fig. 9A,B). Pax-3 and myoD expression are co-expressed in developing limb muscles (Fig. 7F,G) and experiments with quail-chick chimeras have shown the migratory precursors for limb muscles express Pax-3 (Williams and Ordahl, 1994). Our results suggest the loss of these cells is underlying reason for the limb muscle defect in splotch mice.

**DISCUSSION**

In an earlier study, we have shown that the expression patterns of Pax-3 and Pax-6 in the spinal cord are regulated by signals from the notochord and floor plate (Goulding et al., 1993). The observation that Pax-3 expression is dorsally restricted in both somites and neural tube suggested that signals from the midline may also regulate Pax-3 expression in the somitic mesoderm. In this study, we have analyzed the effects that (a) placing an extra notochord beside the segmental plate and (b) removing the notochord from beneath the neural plate has on the development of somites and on the somitic expression of Pax genes.

**Fig. 7.** Pax-3 expression in the limb. Expression of Pax-3 was examined in the developing limb in chick and mouse embryos by whole-mount in situ hybridization. (A) Expression of Pax-3 in a stage 19 chick embryo showing Pax-3-expressing cells in the early limb bud (see arrows). (B) Stage 22 chick embryo showing Pax-3-expressing cells in the limb bud (see arrow). The open arrows show Pax-3-expressing cells at the lateral edge of somites that do not migrate into the limb. (C) Transverse section through stage 22 embryos showing Pax-3-expressing cells in the dorsal limb bud. (D) Transverse section through day 10 mouse embryo forelimb showing Pax-3-expressing cells in proximal regions of the limb. (E-F) Expression of Pax-3 and myoD in the limb. Pax-3 and myoD expression was examined by in situ hybridization in the forelimb of a stage 25 chick embryo. (E) Bright field of panel F. (F) Pax-3 expression. (G) myoD expression. Arrows mark the developing limb muscles. h, humerus; lb, limb bud; m, limb muscles; n, notochord; s, somites; sc, spinal cord; v, vertebra. Bar, 200 μm (A-D); 500 μm (E-G).

Our results show signals from the notochord regulate the pattern of Pax gene expression in somites. In addition, these experiments provide evidence that notochord-derived signals are able to prevent the differentiation of dermomyotome cells and in turn promote the development of sclerotome.

During somite morphogenesis, dorsal cells develop into dermomyotome and ventral cells into sclerotome. The differentiation of sclerotome cells occurs several hours after the somites have formed and can be first seen when cells in the ventromedial regions of the somite lose their epithelial arrangement (Keynes and Stern, 1988). These changes in cell morphology and differentiation appear to result from signaling by the adjacent notochord and neural tube (Rong et al., 1992). Our results show that placing a second notochord beside the segmental plate causes dorsal cells to undergo an epithelial-to-mesenchymal transition characteristic of sclerotome development. This change in the morphology of the somite is closely associated with the inhibition of Pax-3 expression in dorsal regions of the somite adjacent to the ectopic notochord (Figs 3,4). The change in Pax-3 expression in the presumptive dermomyotome in response to an extra notochord occurs early and the ectopic notochord is able to repress Pax-3 expression in the segmental plate and in newly formed somites that still have an epithelial morphology (Fig. 3). Following on from the change to Pax-3 expression, the arrangement of cells in the dorsal somite is altered, now resembling ventromedial cells that have differentiated into sclerotome and indicating that these cells are no longer ‘dermomyotome’. In addition, cells in the vicinity of the second notochord begin to express Pax-9 (Fig. 5). Taken together, these results suggest that the ectopic notochord provides a ventralization signal that is able to direct dorsal cells in the somite toward a ventral phenotype, i.e. sclerotome. As such, the effect of the notochord on the Pax gene expression and on cell differentiation in the mesoderm is very similar to its effects on dorsoventral patterning in the spinal cord (Yamada et al., 1991; Goulding et al., 1993).

The temporal restriction of Pax-3 expression during somite morphogenesis and the ability of notochord to alter Pax-3 expression are consistent with the fate of somitic cells being determined after the somite forms. In experiments where the segmental plate was rotated 180° about the AP axis, the somites developed normally, indicating that cells in the segmental plate have the ability to differentiate into either dermomyotome or sclerotome (Stern et al., 1988). A similar plasticity is seen in the two most recently formed somites, indicating that the decision to become dermomyotome or sclerotome does not occur until after the somites have formed (Aoyama and Asamoto, 1988). Consistent with these findings, we have observed that Pax-3 expression is less responsive to signals from the notochord in somites after they have formed, indicating that the decision to differentiate into dermomyotome may occur close to the time that Pax-3 expression becomes restricted to the presumptive dermomyotome.

In addition to its effects on Pax-3 expression, we have shown that the notochord regulates the expression of Pax-7 and myoD, which are expressed in the dermomyotome and myotome, respectively. An ectopic notochord was able to repress the expression of both genes in the somite and induce the ectopic expression of Pax-9, a gene normally expressed in sclerotome cells (Fig. 5). These results together with those of
Pourquie et al. (1993) suggest that signals from the notochord are able to induce mesoderm cells to differentiate into sclerotome. Previous studies have shown that after removing the notochord the floor plate is lost (van Straaten et al., 1991; Yamada et al., 1991) and a single large myotome often forms beneath the neural tube (Kitchin, 1949). In regions of the embryo lacking a notochord and floor plate, Pax-3, Pax-7 and myoD are expressed in the mesoderm beneath the neural tube in regions where sclerotome normally develops (Fig. 6). At the same time, expression of Pax-9 is conspicuously absent from this region of the embryo (Fig. 6). Our results describing the effect of removing the notochord on Pax gene expression support the hypothesis that the notochord is necessary for the development of sclerotome cells and for the ‘ventralization’ of somites.

The role of the floor plate and neural tube in the patterning of the mesoderm is less clear and a number of experiments suggest that the effects of the notochord on the mesoderm may be distinct from the notochord-induced changes that occur in the neural tube. In many of our grafting experiments, clear differences were seen between the effects of the notochord on Pax gene expression in the mesoderm and in the spinal cord (see Fig. 5A-C). When an ectopic notochord was placed at a distance from the spinal cord, expression of Pax-3 in the dermomyotome was severely disrupted without any significant changes to Pax-3 expression in the spinal cord (not shown).

We have shown that removal of the notochord alone results in an enlarged dermomyotome and no sclerotome, whereas removal of the neural tube and notochord together causes the loss of both myotome and sclerotome (Rong et al., 1992). These results argue that the neural tube and notochord can influence cell differentiation in somites and that neural tube-derived signals may be necessary for myotome differentiation. In our experiments examining myoD expression in embryos where the notochord had been surgically removed, somites positioned some distance from the residual notochord did not express myoD. This suggests signaling from the notochord to the neural tube may be necessary for the induction of myoD in somites.

The close correlation between the changes in Pax gene expression and cell differentiation in response to the described notochord manipulations raises the possibility that the Pax genes can regulate the differentiation of cells in the somitic mesoderm. We have investigated the role of Pax-3 in somitic cell differentiation by analyzing muscle development in splotch embryos. While the dermomyotome appears normal in day 10.5 mutant embryos, the development of the limb and shoulder muscles in older embryos is severely disturbed in four alleles of splotch, Sp, Spd, Sp1H and Sp2H, as assessed by morphology (Franz et al., 1993) and by the expression of myogenin and acetylcholinesterase (Fig. 8). Both the axial and body wall musculature are relatively normal by these criteria, indicating that cells in the myotome still have the capacity to undergo myogenic differentiation and are patterned appropriately. The defect in splotch mice appears to affect disproportionately those cells in the lateral part of the dermomyotome that express Pax-3 at high levels (see Fig. 2). In the chick, these cells have been shown to migrate from the ventral dermomyotome into the limb bud to generate the limb musculature (Williams and Ordahl, 1994). The exact reason for the loss of these cells in splotch mice is unclear. Pax-3-expressing muscle precursors derived from the ventral dermomyotome may no longer migrate into the limb bud or alternatively, limb muscle precursor cells may still invade the limb where they no longer express Pax-3 and as a consequence are unable to differentiate into muscle. The limb phenotype of splotch mice bears comparison with the loss of neural crest-derived tissues found in splotch embryos (Auerbach, 1954). The neural crest defect in splotch mice is believed to arise because neural crest cells are impaired in their migration from the neural tube (Moase and Trasher, 1990). If the limb muscle defect in splotch mice is also one of cell migration, then Pax-3 may regulate the expression of cell surface molecules that are necessary for the migration of these two populations of cells.

The effects of the notochord on Pax-3 expression together with the changes in muscle development in splotch embryos suggest that Pax-3 is involved in the early steps of the myogenic differentiation pathway. Pax-3 may act upstream of the known myogenic differentiation factors to determine a population of muscle precursors for the limb and shoulder muscles. While the above experiments demonstrate a clear role for Pax-3 in the genesis of limb muscles, the function of Pax-3 in the differentiation of other skeletal muscles is still not clear. The observation that axial muscles develop and express myogenic determination factors in splotch embryos indicates that the loss of Pax-3 in these cells does not alter their differentiation. Axial muscles and limb muscles are derived from two distinct lineages (Selleck and Stern, 1991), and consequently mutations to Pax-3 may differentially affect the limb muscle lineage. Alternatively, the closely related gene Pax-7 may be able to compensate for the loss of Pax-3. This is consistent with the observed expression of Pax-7 in the dermomyotome at the same time as Pax-3. Prior to the migration of the limb muscle precursors from somites, Pax-7 appears to be down-regulated in lateral dermomyotome cells from which the limb muscles are derived, but not in cells located more medially that can contribute to the axial muscles. Functional redundancy has also been suggested as an explanation for why mice lacking either myoD or myf-5 gene do not exhibit gross defects in their musculature (Braun et al., 1992; Rudnicki et al., 1992), even

![Figure 8](https://example.com/figure8.png)
though both genes are able to act as powerful promoters of myogenesis in cultured cells (Olson, 1990).

In summary, the results in this paper describe a close correlation between the expression of *Pax-3* in the somitic mesoderm and the differentiation of somitic cells into dermomyotome. The changes to the patterning of the somitic mesoderm in response to notochord manipulations are closely accompanied by changes to the expression of *Pax-7*, *myoD* and *Pax-9*. In addition, we show *Pax-3* regulates the development of a subset of skeletal muscle cells that are derived from the dermomyotome, since limb muscles fail to develop in mice lacking a functional *Pax-3* gene. This raises the possibility that *Pax-3* may be required for the early steps of myogenesis in vertebrate embryos.

The authors would like to thank Bruce Paterson for the gift of the chicken *myoD* cDNA, Joachim Niessing for the gift of the *Pax-9* plasmid and Soo-Pok Yee for the gift of the mouse *myogenin* gene. We would also like to thank Amata Hornbruch and Marc Olivier for excellent technical assistance, Anne Bang for her help with some of the in situ and Amata Hornbruch, Trevor Kilpatrick, Chris Kintner, Greg Lemke, Charles Ordahl and Marc Tessier-Lavigne for their invaluable discussions and comments on this manuscript. This work was supported by grants from the March of Dimes and National Institutes of Health (NS 31978-01) to M. G. and by the Medical Research Council of Great Britain (A. L.).

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(accepted 22 December 1993)

Note added in proof

A paper describing the regulation of Pax-1 by the notochord has recently been published by Brand-Saberi et al. (1993) Anat. Embryol. 188, 239-245. Their results show Pax-1 is regulated in a similar fashion to Pax-9 by the notochord.