Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord

Martyn D. Goulding1,2,3, Andrew Lumsden2,* and Peter Gruss1

1Department of Molecular Cell Biology, Max Planck Institute for Biophysical Chemistry, 3400 Göttingen, Germany
2Department of Anatomy and Cell Biology, United Medical and Dental Schools, Guy’s Hospital, London SE1 9RT, England
3Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA 92037, USA

*Author for correspondence

SUMMARY

Members of the paired box (Pax) gene family are expressed in discrete regions of the developing central nervous system, suggesting a role in neural patterning. In this study, we describe the isolation of the chicken homologues of Pax-3 and Pax-6. Both genes are very highly conserved and share extensive homology with the mouse Pax-3 and Pax-6 genes. Pax-3 is expressed in the primitive streak and in two bands of cells at the lateral extremity of the neural plate. In the spinal cord, Pax-6 is expressed later than Pax-3 with the first detectable expression preceding closure of the neural tube. When the neural tube closes, transcripts of both genes become dorsoventrally restricted in the undifferentiated mitotic neuroepithelium. We show that the removal of the noto-

INTRODUCTION

A number of genes containing a paired box (Pax) genes have been isolated recently from vertebrates (Deutsch et al., 1988; Burri et al., 1989; Walther and Gruss, 1991; Krauss et al., 1991a,b). The Pax genes encode a family of transcription factors that bind to specific DNA sequences via the paired domain and the paired-type homeodomain (Chalepakis et al., 1991; Goulding et al., 1991; Treisman et al., 1991). All of the Pax genes are expressed during embryonic development in spatially restricted cell populations, suggesting a role in pattern formation. Further evidence for the important role of Pax genes in embryogenesis comes from studies showing that three of the Pax genes are mutated in known mouse mutants (Balling et al., 1988; Epstein et al., 1991; Hill et al., 1991). In each mutant, structures in which these genes are normally expressed in the embryo fail to develop correctly (Gruneberg, 1950; Auerbach, 1954; Hogan et al., 1986).

Many of the Pax genes are expressed during early neurogenesis, where they exhibit distinct regional patterns of transcriptional activity (Nornes et al., 1990; Jostes et al., 1991; Goulding et al., 1991; Krauss et al., 1991a,b; Walther and Gruss, 1991). These expression domains often coincide with developing morphological boundaries in the embryonic nervous system. In the early brain, Pax-6 and pax[zf-a] are expressed in discrete regions of the developing diencephalon and telencephalon (Walther and Gruss, 1991; Krauss et al., 1991a). Pax-6 is expressed in the lateral and dorsal neuroepithelium of the telencephalon from day 10 to day 18 p.c. In the diencephalon, Pax-6 is expressed at high levels in the ventral thalamus with a lower level of expression in cells dorsal of the sulcus diencephalus medius (Walther and Gruss, 1991). Pax-6 and pax[zf-a] both exhibit a sharp caudal border of expression coincident with the posterior commissure that marks the midbrain/forebrain boundary. Pax-7 is expressed at later stages of mouse development in the mesencephalon where its anterior border of expression coincides with the midbrain/forebrain boundary (Jostes et al., 1991). In early embryos, pax[zf-b] and Pax-2 are expressed in the caudal mesencephalon (Krauss et al., 1991; G. Dressler and A. Pueschel, personal communication). Both genes are expressed in a band of cells in the anterior rhombencephalic isthmus (Krauss et al., 1991b) as it forms, where they may regulate the early regionalisation of the midbrain.

Several members of the Pax gene family are expressed in dorsoventrally (DV) restricted regions of the developing
spinal cord (Nornes et al., 1990; Plachov et al., 1990; Jostes et al., 1991; Goulding et al., 1991; Walther and Gruss, 1991). In particular, three mouse Pax genes Pax-3, Pax-6 and Pax-7 are expressed in DV restricted regions of the early neuroepithelium in the spinal cord and hindbrain. Pax-3 and Pax-7 transcripts are restricted to cells in the dorsal neural tube (Jostes et al., 1990; Goulding et al., 1991). In day 10-12 p.c. mouse embryos, Pax-6 is expressed at high levels in mid-lateral regions of the spinal cord, with lower levels in dorsal regions (Walther and Gruss, 1991). Pax-6 transcripts are absent from floor plate cells and the adjacent ventrally located neuroepithelial cells. The zebrafish homologue of Pax-6, pax[zf-a], exhibits a similar pattern of expression in the mid-lateral spinal cord of the early embryo (Krauss et al., 1991a; Pueschel et al., 1992).

The spinal cord exhibits a characteristic dorsal-ventral (DV) pattern, with specific classes of neurons differentiating according to their DV position. These dorsoventral differences in neuronal differentiation are reflected in both the cell types generated and in their time of emergence. Motor neurons are born early in the ventral third of the neural tube and eventually come to lie in two longitudinal columns on either side of the floor plate (Hamburger, 1948). Neural crest cells are generated early from the neural folds and dorsal regions of the neural tube and migrate to various sites in the body where they differentiate into a number of neural and non-neural cell types (Anderson, 1989). The neural crest-derived sensory neurons of the dorsal root ganglia in turn send projections to relay neurons in the spinal cord via the dorsal root entrance zone (Altman and Bayer, 1984). In the rat, a population of early developing TAG-1-positive commissural neurons also arise in the alar plate, and their axons project to the contralateral side of the spinal cord via the floor plate (Dodd et al., 1988; Tessier-Lavigne et al., 1988).

Neurogenesis in the rat spinal cord occurs in a general temporal gradient with neurons in the ventral spinal cord becoming postmitotic before those in the dorsal cord. In the rat spinal cord, most motor neurons are generated between embryonic days 11 and 13, while the majority of the neurons in the intermediate gray and the dorsal substantia gelatinosa appear on embryonic days 12-15 and 14-16, respectively (Nornes and Das, 1974). In the chick, most neurons in the spinal cord are born after stage 10 with the exception of a few postmitotic cells in the cervical region (McConnell and Sechrist, 1980). As in the rat spinal cord, neurons are born in a ventral-to-dorsal gradient (Langman and Haden, 1970). However, a small number of postmitotic cells arise in the dorsolateral cord prior to motor neurons. These cells may be an early population of circumferentially projecting neurons described by Holley (1982).

The importance of the notochord in dorsoventral patterning of the neural tube has been highlighted in studies utilising surgical manipulation of early embryos (Kitchin, 1949; van Straaten et al., 1985, 1988; van Straaten and Drukker, 1987; van Straaten and Hekking, 1991; Yamada et al., 1991). Removal of the notochord results in the loss of the floor plate (van Straaten and Hekking, 1991) and the adjacent bilaterally located motor neuron pools (Yamada et al., 1991). As a result, cells throughout the neural tube appear to be left with a dorsal phenotype (Yamada et al., 1991). In notochordless Xenopus embryos produced by UV irradiation, the number of motor neurons is drastically reduced, and these are now positioned at the ventral midline (Clarke et al., 1991). In exogastrulae Xenopus embryos, Xit-neurons fail to develop in the absence of a notochord (Ruiz i Altaba, 1992). Alternatively, implantation of an additional notochord beneath the lateral midline allows for the additional floor plate to be induced (Placzek et al., 1990) along with the generation of motor neurons in presumptive sensory regions in vivo (Yamada et al., 1991). In sum, these studies demonstrate that the notochord plays a crucial role in ventralising the neural tube by inducing cells at the ventral midline to become floor plate cells. The floor plate forms a non-neurogenic region that separates the neural tube into two neurogenic regions along the ventral midline. In addition, the floor plate provides signals that regulate the development of motor neurons (Yamada et al., 1991) and floor plate cells release a diffusible chemotactic molecule that stimulates and directs the growth of commissural axons in vitro (Tessier-Lavigne et al., 1988).

The genetic events that regulate dorsoventral patterning in the spinal cord are largely unknown. The expression patterns of the Pax genes in the early neural tube suggest that these genes may be involved in aspects of early DV patterning. We have isolated and characterised the expression of the chicken homologues of Pax-3 and Pax-6 and have used these to follow the establishment of the DV restricted expression of both genes in the spinal cord. In addition, we have analysed the effect of the notochord and floor plate on the expression of Pax-3 and Pax-6 in the spinal cord by either transplanting a supernumerary notochord beneath the neural plate or by removing a section of notochord. These experiments show that signals emanating from these ventral midline structures regulate the dorsoventral expression of both genes within the spinal cord neuroepithelium.

**MATERIALS AND METHODS**

**PCR amplification of Pax-3 and Pax-6**

**Primers**

- MG33. TGGAAATTCTGGGCAAG(AG)/TA/CT/TA/CTGA/AG/AC
- MG35. CGATCTCGGATA/AG/TGGAT/CT/CT/CTA/AT/AA/CT/TC
- MG36. GAAGATCTTGGG/CG/GGTGGT(CT)AT/ACT/AAT/ACT/GG
- MG37. CTTCTAGATIGTGG/AG/ATGGC/AGCT/CA/CT/CA/CT/G
- MG39. GAGATC/CTGCTG/AG/AC/CT/AG/AC/CC/AC/CT/CT/G

**PCR amplification reactions**

Pax-3 was amplified by PCR from 100 ng of reverse transcribed mRNA from stage 10 chicken embryos using Replinase DNA polymerase (Du Pont). Following a primary amplification of 25 cycles with primers MG36 and MG39, reactions were separated on a 1.2% LMP-agarose gel. A 720 bp band corresponding to the predicted PCR fragment was isolated and amplified for a further 20 cycles with primers MG37 and MG39. These amplified chicken Pax sequences were gel-purified, cloned into Bluescript KS (Stratagene) and sequenced. Pax-6 was amplified using the same strategy and the primers MG33, MG35 and MG36. A 688 bp Pax-6 PCR fragment was isolated and cloned into Bluescript KS.

**Pax-3 and Pax-6 cDNA isolation**

cDNA clones encoding Pax-3 and Pax-6 were isolated from an
embryonic stage 10 chicken cDNA library (Charlesbois et al., 1990) After transfer to Hybond-N membrane filters (Amersham), 1.2×10⁶ clones were screened with 32P-labelled PCR generated probes for Pax-3 and Pax-6 under high-stringency conditions.

DNA sequencing
Restriction fragments derived from the chicken Pax-3 and Pax-6 cDNAs were cloned into either Bluescript KS or m13 mp18/19. Nucleotide sequences were determined from single-stranded or double-stranded DNAs using the dideoxy chain termination method of Sanger et al. (1977).

Embryos
White Leghorn hens' eggs were incubated at 38°C in a humidified forced-draft incubator. Eggs were windowed and staged according to Hamburger and Hamilton (1951).

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 were then incubated for a further 2 days at 90% relative humidity (RH). Embryos were removed on day 4, fixed and embedded in paraffin and sectioned at 7 µm (n=12).

Notochord implantation
Notochords were implanted at specific medial-to-lateral positions under the open posterior neural plate of stage 9-10 embryos. A longitudinal incision was made directly beside the open caudal neural plate at the level of presumptive somite numbers 10-16. Donor notochord pieces (300-400 µm in length) from stage 10-12 embryos were inserted into the slit and manipulated into position at the required lateromedial (ultimately dorsoventral) position next to the neural plate. Eggs were resealed and incubated at 90% RH for a further 2-3 days. Surviving notochord-implanted embryos were fixed and examined at stage 18-21 (n=39). In some experiments, embryos were recovered 6-24 hours after the graft operation and analysed (n=22). Embryos were either embedded in paraffin or in Tissue Tek (Miles Inc.) and sections cut at 7-10 µm. Transverse sections spanning the notochord implants were examined by in situ hybridisation for expression of Pax-3 and Pax-6. Some sections were also stained with Giemsa and for immunohistochemistry.

In situ hybridisation
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 pre-treated for in situ hybridisation. Radioactive in situ probes were transcribed from linearized plasmid using 100 mCi of 35S-UTP and 100 mCi of 35S-CTP. A 182 bp fragment encompassing the homeobox and octapeptide sequences was used for Pax-3, and a 520 bp fragment spanning both the homeobox and paired box was used for Pax-6. Sections were hybridised at 50°C for 18 hours in a humidified chamber. Slides were washed at 42°C in 2×SSC, 50% formamide, 20 mM 2-mercaptoethanol, followed by RNase digestion (15 minutes at 37°C, 20 mg/ml RNase in TE containing 0.5 M NaCl), then washed overnight in 2×SSC, 50% formamide at 37°C. Prior to dehydtration, a final high-stringency wash at 60°C in 2×SSC, 50% formamide was performed. Slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water) and exposed for 10-14 days at 4°C. Following development, slides were counterstained with Giemsa and coverslips applied.

For whole-mount in situ hybridisation, chicken 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. Embryos rehydrated by passing them back through the methanol series were washed twice with PBT and incubated for 1 hour in PBT containing 5% hydrogen peroxide. Embryos were then washed in PBT, treated with proteinase K (20 µg/ml) for 7 minutes and refixed in 0.2% glutaraldehyde, 4% paraformaldehyde in PBS for 20 minutes at room temperature. Embryos were then transferred to hybridisation buffer (50% formamide, 5×SSC pH 4.5, 1% SDS, 50 µg/ml tRNA, 50 µg/ml heparin) and prehybridised for 1 hour at 70°C. Digoxigenin-labelled RNA probes specific for Pax-3 and Pax-6 were added to the hybridisation buffer to a final concentration of 1 µg/ml. Embryos were hybridised overnight at 70°C, followed by washing in 50% formamide, 1% SDS, 2×SSC pH 4.5 at 70°C for 1 hour before non-specific RNA hybrids were removed by digestion with RNase A (100 µg/ml in TE containing 0.5 M NaCl) for 1 hour. Following RNase treatment, embryos were washed in 50% formamide, 2×SSC pH 4.5 at 65°C for 90 minutes. Embryos were washed extensively in TBST (0.14 M NaCl, 10 mM KCl, 25 mM Tris-HCl pH 7.5, 0.5% Tween-20) before overnight incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim) that had been preabsorbed with chick embryo powder. Embryos were again washed extensively with TBST and the alkaline phosphatase activity visualised by reacting embryos with nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2.

Whole-mount in situ embryos were either mounted whole in glycerol or embedded in albumin-gelatin and Vibratome sectioned at 50-60 µm. Sections were mounted and coverslipped before being photographed. All sections were photographed using a Zeiss Axiophot microscope and dark-field/bright-field illumination.

Immunohistochemistry
Cryostat sections (7-8 µm) were cut, collected on gelatin-subbed slides and air dried. Slides were then briefly fixed in 3.5% formaldehyde-PBS, then washed twice in PBS, blocked with 0.01% hydrogen peroxide followed by 10% FCS. Slides were incubated overnight at 4°C with primary antibody SC1, (Tanaka and Obata, 1984) or DM1, (Burns et al., 1991) diluted 1:1 with PBS containing 0.2% Triton X-100. Slides were washed and the second antibody applied (HRP-conjugated rabbit anti-mouse Ig diluted 1:200, Dakopats). HRP reactions were developed using standard procedures.

RESULTS
Isolation and characterization of the chicken Pax-3 and Pax-6 genes
In order to study the establishment of the dorsoventral expression patterns of Pax-3 and Pax-6 in the spinal cord and identify the nature of the signals that regulate this DV restriction of Pax gene expression, we have isolated and characterised the chicken homologues of the mouse Pax-3 and Pax-6 genes. PCR-generated cDNAs for the chicken Pax-3, Pax-6 and Pax-7 genes were obtained by amplifying reverse transcribed poly(A)+ RNA isolated from stage 10 (Hamburger and Hamilton, 1951) chicken embryos.
Degenerate primers to conserved sequences in the paired box and homeobox of each gene were utilised. cDNAs were amplified by using two primer pairs (3 primers) for each gene in order to amplify unambiguously the homologous genes from the chicken (see Fig. 1 for the oligonucleotides used). The identities of the amplified Pax genes were determined by DNA sequencing.

A full-length cDNA encoding the chicken Pax-3 and a partial cDNA containing the paired box and homeobox sequences of the chicken Pax-6 gene were isolated from a stage 10 chicken cDNA library using the PCR-generated cDNAs as probes under high-stringency conditions (see Fig. 1). The paired box and homeobox sequences of the chicken Pax-3 and Pax-6 genes were determined from these cDNAs. The sequence of the paired domain and homeodomain of the chicken Pax-3 and Pax-6 proteins are shown in Fig. 2. Comparison of the paired domain and homeodomain of both proteins reveals a high degree of sequence similarity with other vertebrate Pax proteins. The chicken Pax-3 and the Pax-6 proteins both share extended regions of complete identity with their murine and zebrafish counterparts. The chicken and mouse Pax-6 paired domains are identical, while three amino acid differences are found in the paired domain of Pax-3. These three amino acids are encoded by the third exon and reside in the less conserved C terminus of the paired domain. The chicken Pax-3 protein contains an alanine instead of a proline in a region predicted to be between the second and third helices. In addition, two amino acids are altered in the putative third helix of the paired domain. One of these, an arginine to lysine exchange, is conservative, the other, a glycine to alanine, is not. Interestingly, both residues have been altered to the two amino acids found in these positions in the mouse Pax-1 and human HuP48 proteins (Deutsch et al., 1988; Burri et al., 1989). The chicken and mouse Pax-3 proteins both show a similar pattern of binding to the e5 sequences and mutated versions of the e5 sequence (M. G., unpublished results). This is consistent with recent studies showing the mouse Pax-1 and mouse Pax-3 paired domains show the same DNA contacts with the e5 sequence (Chalepakis et al., unpublished data). While no differences in binding to the e5 or related sequences are apparent, the effect of these amino acid exchanges on protein activity in vivo are not known.

The observation that the Pax-6 genes from three widely divergent species encode identical paired-type homeodomains (Krauss et al., 1991a; Walther and Gruss, 1991; Pueschel et al., 1992; this study) underlines the high degree of sequence conservation that has been retained by the vertebrate Pax protein homologues. All of the paired-type homeodomains isolated to date contain a serine residue at the ninth position of helix 3 (Fig. 2). This residue has been shown to be essential for sequence-specific binding and sequence discrimination by the paired-type homeodomain (Treisman et al., 1989). Because of their strict sequence conservation, it seems likely that the chicken and mouse Pax-3 homeodomains recognise and bind the same sequences, as do the mouse and chicken Pax-6 homeodomains. However, it is not known if the different paired-type homeodomains are able to recognise the same or similar DNA sequences in the embryo.

The homology outside of the two DNA-binding domains is also particularly striking. The stretch of 68 amino acids between the two DNA-binding domains is identical in both the chicken and mouse Pax-6 proteins (not shown). Both Pax-3 proteins also share a very high degree of homology outside of the two DNA-binding domains. In the highly charged region of Pax-3 that separates the paired and homeodomains and includes the octapeptide sequence, only a single conservative amino acid exchange is present. The N and C termini of both proteins are also very similar, with the C termini of both proteins being 93% identical (not shown).

**Expression of Pax-3 and Pax-6 in the chicken spinal cord**

Previous studies in the mouse have not addressed how the DV restriction of Pax gene expression in the spinal cord comes about. In the mouse, Pax-3 transcripts were first detected in day 8.5 embryos in the closing neural tube (Goulding et al., 1991). Similarly, expression of Pax-6 in the embryonic mouse spinal cord was first seen on day 8.5 after the neural tube had closed (Walther and Gruss, 1991). To gain a more complete understanding of how these DV restricted patterns of expression emerge, the expression of both genes was analysed in stage 7-11 chicken embryos. An advantage of using the chicken for these studies is that the formation of the neural plate and neurulation are readily observable.
**Paired Domain**

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<tr>
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<td>Mouse Pax-6</td>
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**Homeodomain**

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<td>Mouse Pax-6</td>
<td>LQHRSTRQETQELSLSRTSTRTSTTSTTSQQVESQVEQ</td>
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</table>

**Paired Domain**

`Pax-3` and `Pax-6` are both expressed before the neural tube closes (Figs 3A-E, 4B-D,H). `Pax-3` is detected as early as stage 7, with a high level of expression in the primitive streak region (not shown). In stage 8 embryos, transcripts of `Pax-3` (Fig. 3B) and `Pax-6` (not shown) are found in the neuroectoderm, adjacent to the first developing somites. In these regions `Pax-3` transcripts are confined to cells in the dorsal neural folds, while `Pax-6` transcripts are present in mid-lateral regions of the neural groove. `Pax-3` expression is also seen in caudal regions of the embryo in the neural folds of the neural plate and in the primitive streak caudal to Hensen’s node (Figs 3A, 4A).

The expression patterns of both genes were examined in stage 10-11 embryos (Figs 3, 4). As in earlier embryos, `Pax-3` transcripts are confined to cells at the lateral edge of the neural plate prior to closure of the neural tube (Fig. 4B-D). In the closing neural tube, these `Pax-3`-expressing cells are positioned at the tips of the folds and come to lie adjacent to each other, when the dorsal lips of the neural tube fuse. In the closed neural tube, `Pax-3` is initially expressed only in the dorsal most cells of the neural tube, the roof plate included (Fig. 4E). Following the closure of the neural tube a progressive ventral shift in the expression of `Pax-3` occurs. The progressive dorsal-to-ventral expansion of `Pax-3` expression continues until a ventral boundary of expression is established midway between the floor plate and roof plate and is seen in sections taken 4-5 somite-widths anterior to the point of neural tube closure (Fig. 4F). This ventral limit of `Pax-3` expression in the ventricular zone remains until the mitotic neuropilithedum recedes later in development. In the mouse, the ventral border of `Pax-3` appears to coincide with the sulcus limitans (Goulding et al., 1991). However, it is unclear whether the same is true for the chick, since this feature is not readily observable.

In stage 10-11 embryos, transcripts of `Pax-6` are not detected in the caudal-most regions of the neural plate (Figs 3, 4). Instead `Pax-6` transcription begins just prior to closure of the neural tube. After closure of the neural tube, `Pax-6` is expressed in cells throughout the neural tube, with the exception of floor plate cells and roof plate cells (Fig. 4I). The DV restriction of `Pax-6` expression in the spinal cord develops after neural tube closure, with expression in the ventricular zone being progressively down-regulated in cells adjacent to the floor plate and roof plate (Fig. 4I-M). This results in the generation of a band of `Pax-6`-expressing cells in the mid-lateral region of the spinal cord. Taken together, these results indicate that the expression of `Pax-3` and `Pax-6` begins before the neural tube closes and the expression of both genes in the early neuroepithelium is plastic. It is only after the neural tube closes that their expression domains become dorsoventrally defined.

In both the chicken and mouse, `Pax-3` and `Pax-6` are expressed in the ventricular zone in mitotically active progenitor cells from which the neurons and glia of the nervous system are derived. The observation that the expression of `Pax-3` and `Pax-6` are dorsoventrally restricted in the spinal cord soon after closure suggested that the establishment of these restricted expression domains might precede or accompany the early differentiation of neurons within the spinal cord. Since motor neurons are amongst the first neurons to develop in the spinal cord, the expression of `Pax-3` and `Pax-6` was examined in relation to the emergence of motor neurons. In brachial regions of the spinal cord, motor neurons begin to be born at stage 15 (Hollyday and Hamburger, 1977). The homeodomain protein `Islet 1` is also expressed in early differentiating motor neurons (Ericson et al., 1992). `Islet-1`-positive cells first appear in the brachial spinal cord between stages 15 and 16. We have analysed...
the expression of Pax-3 and Pax-6 in the brachial region prior to this, in stage 14 embryos, to determine the DV expression patterns of both genes immediately prior to the birth of motor neurons. In the stage 14 chick spinal cord, Pax-3 expression was restricted to the dorsal neuroepithelium, while Pax-6 was expressed in a band of cells in the midlateral neural tube (Fig. 5B,D). The patterns of Pax gene expression in stage 14 embryos, closely resemble the expression patterns seen at later stages of development (cf Figs 5 and 6).

Sections immediately adjacent to those used for the in situ analysis were stained with the monoclonal antibodies SC1 and DM1 to identify motor neurons. In the chicken embryo these antibodies recognise a $100\times10^3 \, M_r$ protein present in motor neurons, notochord and floor plate cells (Tanaka and Obata, 1984; Burns et al., 1991). In stage 14 embryos, no staining of floor plate cells or motor neurons was detected, although staining of the notochord was seen with both antibodies (Fig. 5A,C). This lack of staining of cells in the ventral neural tube with the SC1 and DM1 anti-
Fig. 4. Expression of Pax-3 and Pax-6 in early spinal cord development. The emerging patterns of Pax-3 and Pax-6 in the spinal cord were examined in stage 10-11 embryos by whole-mount in situ hybridisation. Embryos were embedded in albumin-gelatin and 30 μm sections cut. (A) Section through the primitive streak region of a stage 8 embryo showing expression in the epiblast and migrating cells in the primitive streak. (B-F) Transverse sections through a stage 10 embryo, showing the developing expression of Pax-3 in the spinal cord. Pax-3 expression is restricted to cells in the neural folds until the neural tube closes. Soon after closure, Pax-3 is expressed throughout the dorsal half of the spinal cord. (G-L) Transverse sections through a stage 11 chick embryo showing Pax-6 expression in the developing spinal cord. Pax-6 expression begins just before the neural tube closes in a broad band of cells. Expression of Pax-6 is then progressively restricted to the mid-lateral neuroepithelium. The relative positions of the transverse sections in the embryo are indicated. PS, primitive streak; N, notochord; NP, neural plate; DM, dermomyotome.
bodies is consistent with earlier studies showing that motor neurons do not develop in this region until after stage 15 (Hollyday and Hamburger, 1977; Ericson et al., 1992). Consequently, the DV restriction of Pax-3 and Pax-6 in the spinal cord appears to precede the differentiation of motor neurons.

Altered Pax gene expression following notochord removal

The effects of the notochord on the expression of Pax-3 and Pax-6 in the spinal cord were examined by either removing the notochord from part of the embryo or implanting a second notochord immediately adjacent to the closing neural plate. Removal of a segment of notochord 3-4 somites in length from beneath the caudal neural plate of stage 10 embryos, resulted in marked morphological changes to the neural tube. In sections taken 50-80 μm distal to the residual notochord, no morphologically distinguishable floor plate was seen (Fig. 6D). In some sections, dorsal root ganglia were present and of normal size, but were positioned more ventrally with respect to the spinal cord (not shown). No ventral roots were discernable, in line with previous studies showing that motor neurons fail to develop in the ventral spinal cord following extirpation of the notochord (Yamada et al., 1991). Cell counts from regions from which the notochord had been removed showed that the neural tube contained 15-21% fewer cells in the ventricular zone than in equivalent unoperated embryos. In sections taken midway along the region from which the notochord had been removed, the spinal cord was reduced in size, the floor plate was absent and dorsal root ganglia were either very small and abnormal or completely missing (Fig. 6G).

Dramatic changes in the expression patterns of both Pax-3 and Pax-6 were seen as a result of removing the notochord. In regions of the embryo lacking a notochord, the expression of Pax-3 in the spinal cord extended further ventrally (Fig. 6E,H). In sections taken 50-80 μm from the residual notochord, Pax-3 transcripts were present in all but the ventralmost cells of the neuroepithelium (Fig. 6E). In sections immediately adjacent to these, Pax-6 expression extended into the very ventral part of the spinal cord (Fig. 6F). These regions normally do not express Pax-6 and give rise to floor plate cells and motor neurons. Both of these cell types appear to be absent in these sections. Interestingly, where motor neurons normally develop, a band of cells in which both genes are expressed at high levels was seen. These cells may be analogous to cells found in the normal embryo, only positioned more dorsally (c.f. Fig. 6B,C).

Transcripts of Pax-3 were detected in cells throughout the entire neural tube with the exception of the roof plate in sections 90 μm or further from the residual notochord (Fig. 6E). In these sections, cells expressing high levels of Pax-3 were present in the ventral spinal cord where neurons and the floor plate cells normally develop (Fig. 6H). In adjacent sections, little or no expression of Pax-6 expression was observed (Fig. 6I). The absence of signals from the notochord results in cells throughout the spinal cord expressing Pax-3. In contrast, Pax-6 is either no longer expressed or expressed at very low levels in these cells. Together these results suggest that signals emanating from the notochord are required for the correct spatial restriction
Fig. 6. The effect of notochord removal on the expression of *Pax-3* and *Pax-6*. Notochords were surgically removed from stage 10 embryos. Embryos were allowed to develop for a further two days before being recovered. Sections (7 µm) were cut and analysed by in situ hybridisation for expression of *Pax-3* and *Pax-6*. (A-C) Morphology and expression of *Pax-3* and *Pax-6* in the normal embryo. The spinal cord is outlined. (A) Bright-field, (B) dark-field showing expression of *Pax-3*. (C) Dark-field showing expression of *Pax-6*. (D-F) Morphology and expression of *Pax-3* and *Pax-6* in transverse sections in notochordless embryos. These sections were taken 50-80 µm from the residual notochord. The ventricular zone of the spinal cord is outlined in E and F. (D) Bright-field, (E) dark-field showing expression of *Pax-3*. (F) Dark-field showing expression of *Pax-6*. (G-I) Morphology and expression of *Pax-3* and *Pax-6* in the spinal cord following notochord removal. Sections were taken >90 µm from the unoperated notochord. (G) Bright-field, (H) dark-field showing expression of *Pax-3*. (I) Dark-field showing expression of *Pax-6*. D, dermatome; F, floor plate; N, notochord; R, roof plate; S, spinal ganglion; VR, ventral root; X, site of notochord removal. The arrows in G mark axons projecting from the neural tube. The arrows in E and F indicate cells expressing both genes.
Fig. 7
Fig. 7. Effect of the notochord on Pax gene expression and motor neuron differentiation. Pieces of notochord (300-400 µm in length) were implanted beneath the neural plate of stage 10 chick embryos. Development was continued by incubating embryos at 37.6°C for a further 2 days (E,F). Embryos were fixed and serial cryostat sections (8 µm) spanning the site of the notochord implant were collected. Slides were either stained with the monoclonal antibody SC1 or hybridised with probes specific for either Pax-3 and Pax-6. (A,D,G) SC1 expression in the spinal cord of a stage 21 embryo showing the induction of ectopic motor neurons and floor plate by the grafted notochord. Motor neurons and floor plate cells are indicated by arrows. In adjacent sections (B,E), the ectopic floor plate (F′) can also be seen. (C,H) Expression of Pax-3. (F,I) Expression of Pax-6. The two white bars in I indicate the relative band-width of Pax-6-expressing cells. F, floor plate; F′, induced floor plate; N, notochord; N′, grafted notochord; S, spinal ganglion.

of Pax-3 and Pax-6 in the spinal cord and, in the case of Pax-6, may be necessary for expression. Both genes are normally restricted to the neuroepithelial layer of the spinal cord (Fig. 6B,C). Removal of the notochord does not result in the expression of Pax-3 in postmitotic cells. This suggests that the removal of the notochord does not result in the complete de-regulation of Pax gene expression in the spinal cord, rather that signals from the ventral midline act only to restrict gene expression to defined DV regions of the neuroepithelium.

Effect of a supernumerary notochord on Pax gene expression

The effect of an additional notochord on the development of the neural tube was examined by implanting a 300-400 µm length of notochord under the lateral neural plate of stage 9-10 embryos. Grafts were placed immediately adjacent to the neural plate in the brachial region of the embryo (presumptive somites 10-16). Embryos were removed from the egg on day 4 and the graft site was examined morphologically to determine the effect of the notochord on the spinal cord. Sections through the region of the graft showed that the spinal cord was reorganised in response to the grafted notochord. Grafting the supernumerary notochord medially, close to the host notochord, resulted in either an enlarged floor plate or the development of a second floor plate close to the normal floor plate (not shown). More dorsal placement of the notochord resulted in the development of an additional floor plate in lateral regions of the spinal cord (Fig. 7). This was accompanied by the development of an ectopic column of motor neurons in the dorsal region of the spinal cord (Yamada et al., 1991, Fig. 7).

The expression of both Pax-3 and Pax-6 genes within the spinal cord changed in response to the additional notochord. A dorsal shift in the expression domains of both genes occurred in regions of the spinal cord adjacent to where a second notochord had been placed. In each case, the extent of the dorsal shift in the ventral border of Pax-3 and Pax-6 expression closely followed the DV position of the grafted notochord. When the extra notochord occupied a ventral position, very little difference was seen in the expression patterns of both genes (not shown). When the notochord was implanted in a more lateral (ultimately dorsal) position, cells in the ventricular zone expressing Pax-3 and Pax-6 were positioned further dorsally (Fig. 7). These changes to the expression were found only in the side of the cord bearing the graft and in regions immediately adjacent to the implanted notochord. In embryos where the notochord graft was positioned dorsally, the expression domains of both genes were severely compressed on the side of the implant. This is most apparent in Fig. 7, where the number of cells expressing a high level of Pax-6 was reduced from a band 20-25 cell bodies wide to a band only 8-10 cells wide (see Bars, Fig. 7).

Alterations to gene expression and cell phenotype

The changes to the expression domains of both genes following implantation of a supernumerary notochord were compared with the distribution of motor neurons and floor plate cells in the spinal cord using the SC1 antibody (Fig. 7). Adjacent cryostat sections through the graft site were processed either for immunohistochemistry or in situ hybridisation. In these experiments, the grafted notochord (N) was placed dorsally, resulting in the induction of an ectopic floor plate (F′) and dorsally positioned ectopic motor neurons (Fig. 7E,F). In regions of the neural tube adjacent to the ends of the donor piece of notochord, a broadening of the band of motor neurons was observed on the side of the spinal cord bearing the graft (Fig. 7G). This effect was observable up to a distance of 50-60 µm from the end of the implanted notochord. In sections immediately adjacent to those examined by SC1 immunohistochemistry, transcripts of Pax-3 and Pax-6 in the spinal cord were present only in dorsal cells on the side of the embryo bearing the supernumerary notochord (Fig. 7B,C).

The changes to the expression patterns of both genes appear to parallel the appearance of ectopic motor neurons and an ectopic floor plate that occur in response to the notochord graft. On both the unoperated and operated sides of the spinal cord, Pax-6 appears to be expressed in cells in the ventricular zone that lie just dorsal to cells expressing SC1 (Fig. 7E,F,G,I). This suggests that changes in the expression patterns of the Pax genes in response to a grafted notochord occur in a direction that reflects the later emergence of extra motor neurons in response to the grafted notochord.

Changes to Pax gene expression occur early

The above experiments demonstrated that the expression of Pax-3 and Pax-6 in stage 19-21 embryos is drastically altered after grafting a supernumerary notochord beneath the neural plate. Previously, the effects of the notochord on neuronal phenotype (Yamada et al., 1991) and Islet-1 expression in motor neuroblasts (Ericson et al., 1992) have been analysed in stage 20-25 embryos. The first motor neurons are born in this region at stage 15 (Hollyday and Hamburger, 1978) and by stage 20 the intermediate zone of the spinal cord contains large numbers of postmitotic neuroblasts, many of which have undergone differentiation into specific classes of neurons. While Pax-3 and Pax-6 are expressed in the ventricular neuroepithelium suggesting expression in neural progenitor cells, there remained a possibility that the changes to Pax gene expression seen in
embryos recovered 40-48 hours after operating (stage 19-21) were only late events in the repatterning of the chick spinal cord.

To ascertain whether changes to Pax gene expression occur early, before motor neurons differentiate, embryos into which a notochord had been grafted were analysed 6-24 hours after surgical manipulation. Pieces of notochord, 200-300 µm long, were inserted into stage 10-11 embryos in the anterior region of the neural plate prior to neural tube closure, and embryos were recovered 6-24 hours later. In sham-operated stage 10 embryos, which were fixed and examined at stage 14, Pax gene expression was unaltered, and no changes in the shape and morphology of the spinal cord were seen (not shown). In embryos into which a piece of ectopic notochord had been grafted beneath the closing neural plate, distinct differences in Pax-3 and Pax-6 expression were seen as early as 6 hours (not shown). Fig. 8 represents a typical result from such an operation in embryos recovered 9 hours after operating. The grafting of a supernumerary notochord appears to prevent the ventral shift in Pax-3 expression that normally occurs at these stages of spinal cord development (c.f. Fig. 4). This results in the establishment of a ventral boundary of Pax-3 expression at a more dorsal position in the spinal cord (Fig. 8B). Pax-6 expression appears to be directly repressed in cells adjacent to the notochord graft, resulting in the restriction of Pax-6 expression to the dorsal neural tube (Fig. 8D). The results of these and other grafting operations demonstrate that the changes to Pax-3 and Pax-6 expression in the neural tube occur rapidly in response to the grafted notochord. The resultant changes in Pax gene expression in neuroepithelial cells appear to coincide with the first detectable changes in

Fig. 8. Early effects of the notochord on Pax-3 and Pax-6 expression in the spinal cord. Pieces of notochord were grafted beneath the neural plate of stage 10-11 embryos. Embryos were allowed to develop for a further 9 hours. Pax-3 and Pax-6 expression was detected by whole-mount in situ hybridisation. Transverse vibratome sections (50 µm) were cut to visualise expression in the spinal cord. (A,B) Expression of Pax-3 in whole-mounted in situ. (A) Expression of Pax-3 in a transverse section through a stage 12-13 embryo lacking a notochord implant. (B) Expression of Pax-3 in a transverse section through the region of the same embryo containing a supernumerary notochord. (C,D) Expression of Pax-6 in whole-mounted in situ. (C) Expression of Pax-6 in a transverse section through a stage 12-13 embryo. (D) Expression of Pax-6 in a transverse section through the region containing the supernumerary notochord. N, notochord; N', notochord implant.
the morphology of the neural tube, namely the wedging of the neuroepithelium adjacent to the notochord.

Effect of the floor plate on Pax gene expression

Previously it has been shown that grafting a piece of floor plate beside the neural plate results in the induction of an ectopic floor plate and the generation of extra motor neurons (Placzek et al., 1990; Yamada et al., 1991). We have analysed whether the floor plate independently of the notochord is able to regulate the expression of the Pax genes in the spinal cord. When a piece of floor plate was grafted beside the mid-lateral neural plate of stage 9-10 embryos, a reorganisation of the spinal cord on the side of the graft was seen in embryos examined at stage 14 (Fig. 9). Expression of both Pax-3 and Pax-6 on the side of the graft was more dorsally restricted than on the unoperated side indicating that the floor plate can mimic the effects of the notochord on Pax gene expression (Fig. 9B,C). These changes in gene expression at stage 14, in embryos into which a piece of floor plate had been grafted, closely resembled the changes seen in stage 14 embryos when a notochord was grafted underneath the neural plate at stage 10 (not shown). In stage 14 embryos, weak expression of Pax-6 was seen sometimes in a ventral band of cells on the operated side of the neural tube (Fig. 9C). However, this expression was not seen in embryos killed at later stages of development.

DISCUSSION

We have isolated and characterised the homologues of the Pax-3 and Pax-6 genes from the chick. Both genes encode proteins that share a high degree of similarity with their murine homologues. The chicken Pax-6 protein appears identical to the mouse Pax-6 protein in the paired domain, homeodomain and intervening 68 amino acid region, while the chicken and mouse Pax-3 genes show an overall identity of 98%. The sequence conservation outside of the two DNA-binding domains is striking in both proteins. In the Pax-3 protein, two domains known to regulate transcriptional activity (Chalepakis et al., unpublished data) have been conserved suggesting that the mouse and chicken proteins may have very similar transcriptional activities in the embryo. In addition to the remarkably high conservation of protein structure, the spatial and temporal expression patterns of these genes in embryos of different vertebrate species are strikingly similar. The sequence conservation outside of the two DNA-binding domains is striking in both proteins. In the Pax-3 protein, two domains known to regulate transcriptional activity (Chalepakis et al., unpublished data) have been conserved suggesting that the mouse and chicken proteins may have very similar transcriptional activities in the embryo. In addition to the remarkably high conservation of protein structure, the spatial and temporal expression patterns of these genes in embryos of different vertebrate species are strikingly similar. The spatial distribution of Pax-3 transcripts in the early chicken embryo closely matches the expression of the mouse Pax-3 gene (Goulding et al., 1991), with expression in the neural crest, dorsal spinal cord and dermomyotomal cells. The chicken Pax-6 gene also exhibits a very similar pattern of expression to the mouse Pax-6 and zebrafish pax[zf-a] genes (Walther and Gruss, 1991; Krauss et al., 1991a) suggesting that the mechanisms regulating the expression of these genes in the embryo have been conserved in vertebrates.

In this study, we have begun to analyse the tissue interactions that are responsible for generating the dorsoventrally restricted patterns of Pax gene expression in the spinal cord. The early phase of Pax-3 expression in the spinal cord

Fig. 9. Early effects of the floor plate on Pax gene expression. Pieces of floor plate (150-200 µm in length) from stage 11-12 embryos were grafted into stage 10 host embryos beside the closing neural plate. Embryos were incubated for a further 20 hours until they had developed to stage 14-15. Embryos were isolated, fixed and serial cryostat sections through the graft site were collected. Sections were then analysed for Pax-3 and Pax-6 expression by in situ hybridisation. In situ hybridisation of an embryo into which a supernumerary floor plate has been grafted (F'). Embryos were recovered 20 hours after the operation. (A) Bright-field of adjacent section. N.B. The floor plate in this section is broader than the sections depicted in the dark-field photomicrographs. (B) Dark-field photomicrograph showing Pax-3 expression. (C) Dark-field showing Pax-6 expression. The notochord (N) is marked in all dark-field photomicrographs as well as the position of the roof plate. N, notochord; F, floor plate; F', induced floor plate; R, roof plate; DM, dermomyotome.
suggest two signals may control the regional expression of Pax-3 in the spinal cord. A signal from dorsal cells that progressively activates expression within the neuroectoderm in a dorsal-to-ventral sequence, and a notochord-derived signal that represses Pax-3 expression in a distance-dependent manner. The effects of the notochord manipulation on the expression of Pax-3 in the spinal cord are consistent with this model. When the notochord is removed from beneath the neural plate, Pax-3 is expressed ectopically in ventral neuroepithelium (Fig. 6). Alternatively, when a supernumerary notochord is placed beside the neural plate, cells adjacent to the implanted notochord are prevented from expressing Pax-3 (Figs 7, 8). This is also consistent with the observation that a ventrally positioned notochord causes a small shift in Pax expression, while placing the notochord in more dorsal positions results in the dorsal restriction of Pax gene expression.

Pax-6 expression in the spinal cord develops differently from Pax-3. Pax-6 expression begins later than Pax-3, initially being expressed in a broad band of cells in the closing neural plate (Fig. 4I). Pax-6 expression is then progressively repressed in both dorsal and ventral regions of the spinal cord resulting in the expression of Pax-6 in intermediate regions of the spinal cord. These results suggest Pax-3 is repressed in ventral and dorsal neuroepithelial cells. The repression of Pax-6 expression in neuroepithelial cells adjacent to the grafted notochord may be similar to the down regulation of Pax-6 in the ventral spinal cord that occurs normally (cf. Figs 4 and 8).

The results of the notochord removal experiments are a little harder to interpret, since Pax-6 is only expressed at very low levels in dorsal regions of the spinal cord (Figs 5D, 6C). Previously, Yamada et al. (1991) have shown that removal of the notochord results in the expression of a dorsal antigen, AC4, the ventral spinal cord. In the spinal cord, Pax-3 is expressed in neuroepithelial cells with a dorsal identity. The observation that Pax-3 is expressed ectopically in the ventral cord following notochord removal suggests that these precursor cells may have acquired a dorsal phenotype. It is possible that the absence or very low level of expression of Pax-6 in the neuroepithelium that occurs following notochord removal also reflects this change in cell phenotype, since cells in the dorsal spinal cord normally express very low levels of Pax-6. Alternatively, expression of Pax-6 may depend on signals from the notochord and floor plate or a population of cells that are lost following the removal of the notochord.

The expression of Pax-3 and Pax-6 are early events in the genesis of DV patterning within the spinal cord with their dorsovenously restricted expression patterns appearing before the differentiation of most neurons (Fig. 5). The notochord-induced changes to the expression of both genes in the spinal cord also occur early and show a close correlation with the changes to cell phenotype (Yamada et al., 1991) or Islet-1 expression in motor neuroblasts that have been previously described (Ericson et al., 1992). Our finding that changes to Pax-3 and Pax-6 occur in undifferentiated neuroepithelial cells in response to the notochord demonstrates that signals from the notochord can alter gene regulation in progenitor cells and do not act just as differentiation signals. This raises the possibility that the effects of the notochord on the expression of genes such as Pax-3 and Pax-6 in neural progenitor cells may influence their subsequent pathways of differentiation.

The above studies have described the expression of Pax-3 and Pax-6 in the developing spinal cord and the effect of the notochord and floor plate on the regional expression. Functional analysis of the Pax proteins show they act as specific transcription factors in vitro (Goulding et al., 1991; Chalepakis et al., 1991; Chalepakis et al., unpublished data; C. Walther, G. Chalepakis and P. Gruss, unpublished data) and in vivo (Adams et al., 1992). The fact that Pax-3 and Pax-6 encode transcription factors makes it likely that the notochord-induced changes in Pax gene expression lead to further changes in the genetic program of progenitor cells and their progeny. It remains to be seen whether these changes to Pax gene expression in progenitor cells are able to influence the later changes in cell differentiation that occur in response to the notochord.

The nature of the signals from the notochord and floor plate responsible for the changes in the expression of Pax-3, Pax-6 and Islet-1 (Ericson et al., 1992) in the spinal cord are unknown. It is unclear whether signalling from the notochord involves a diffusable morphogen or is mediated by cell-to-cell signalling. In vitro experiments suggest that cell contact is required for the notochord to induce a floor plate (Placzek et al., 1990, 1993). In addition to the signals emanating from the ventral midline structures, dorsal structures such as the roof plate may also provide signals, some of which might be responsible for the dorsal-to-ventral sequence of Pax-3 transcriptional activations in the dorsal spinal cord.

Recently, a number of mutations affecting the mouse homologues of Pax-3 and Pax-6 have been described. Mutations to Pax-3 give rise to the splotch phenotype (Auerbach, 1954; Epstein et al., 1991; Goulding et al., unpublished data), while the Pax-6 gene is mutated in Small eye mice (Hill et al., 1991). To date, the analysis of both mouse mutants has not provided any clear evidence for the involvement of either gene in the dorsovenous patterning of the spinal cord. The loss of Pax-3 or Pax-6 function appears not to drastically alter the structure of the spinal cord. No severe defect has yet been observed in the spinal cord of Small eye mice; however these studies have not analysed in any detail the patterning of neurons within the spinal cord (B. Hogan, personal communication). A number of defects are present in the spinal cord of splotch embryos. Fewer differentiated cells are found in dorsal regions of the spinal cord of embryonic d13 splotch mice and these appear less organised than their normal siblings (M. G., unpublished results). The peripheral nervous system in the trunk region is severely affected. In Sp heterozygotes and SpD homozygotes, dorsal root ganglia are smaller in size and, in Sp homozygotes, they are often completely absent (Auerbach, 1954; Moase and Trasler, 1989). The defects observed in the spinal cord and peripheral nervous system of splotch mice suggests Pax-3 plays a role in spinal cord development. However, it is unclear whether changes to DV patterning underly these defects in the spinal cord. The differences in the migration of neural crest cells in vitro that have been described may be in part responsible for the loss of melanocytes and other neural crest-derived cell types in
splotch mice (Moase and Trasler, 1990). Since little is known of the changes that occur at the cellular level in either mutant, further analysis of both mutants will be required to ascertain the role, if any, that both genes play in the DV patterning of the spinal cord.

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