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Inhibition of floor plate differentiation by *Pax3*: evidence from ectopic expression in transgenic mice

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SUMMARY

The Pax genes containing a complete paired-type homeobox are expressed in restricted dorsoventral domains of the undifferentiated neuroepithelium. Their expression respond to signals that pattern the neural tube and which emanate from the notochord, floor plate and overlying ectoderm. In order to determine whether the dorsally restricted *Pax3* gene can influence the fate of cells within the neural tube, we produced transgenic mice over-expressing *Pax3* in the entire neural tube under the *Hoxb-4* promoter/region A enhancer. In two distinct transgenic mouse lines, we observed embryos with abnormal limb, eye, brain and neural tube development. Expression of *Pax3* was not sufficient to dorsalize cells from the ventral intermediate zone, which still expressed ventral markers. However, in these embryos, expression of *Pax3* at the ventral midline was associated with the absence of floor plate differentiation. Under these conditions, motor neurons still differentiated in the ventral spinal cord, although in fewer numbers.

Key words: Pax genes, transgenic mice, neural tube, floor plate, notochord, holoprosencephaly, polydactyly, mouse

INTRODUCTION

During the development of the nervous system, the relative position of the neural precursors along the rostrocaudal and dorsoventral axis is a major determinant of their final differentiation fate. In the neural tube, motor neurons differentiate early and settle ventrally as bilateral columns. Other cell types such as commissural neurons and interneurons differentiate later in the dorsal region. This dorsoventral patterning within the neural tube depends on factors produced at the ventral midline and by the overlying non-neural ectoderm (for a review: Placzek, 1995).

The notochord appears to be the source of an early contact-dependent and diffusible factor that induces the differentiation of the floor plate at the neural tube ventral midline and of motor neurons bilaterally (van Straaten et al., 1985, 1988; van Straaten and Hekking, 1991; Placzek et al., 1990, 1993; Bovolenta and Dodd, 1991; Yamada et al., 1991, 1993; Goulding et al., 1993a). During this process, the floor plate acquires similar inducing activities. Recent evidence indicates that the gene *Sonic hedgehog* (*Shh*) is involved in these events. Firstly, the distribution of *Shh* transcripts correlates with this ventralizing activity (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994, 1995; Marti et al., 1995a). Secondly, misexpression of *Shh* leads to the expression of floor plate markers in ectopic position (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Ruiz i Altaba et al., 1995a). Thirdly, recombinant Shh peptides or COS cells expressing them induce the differentiation of floor plate cells and of motor neurons in vitro (Roelink et al., 1994, 1995; Marti et al., 1995b; Tanabe et al., 1995). The winged-helix proteins Pintallavis (frog) and HNF3β (mouse) also appear to mediate floor plate differentiation as their expression in the neuroepithelium is sufficient to induce the differentiation of floor plate cells (Ruiz i Altaba et al., 1993; Ruiz i Altaba et al., 1995b; Sasaki and Hogan, 1994).

Similarly, the acquisition of dorsal fate appears to be initiated at the neural plate stage by short-range signals from the overlying surface ectoderm (Moury and Jacobson, 1989; Dickinson et al., 1995; Liem et al., 1995; Selleck and Bronner-Fraser, 1995). Two members of the *TGFβ* gene family, *BMP4* and *BMP7*, are candidates for this dorsalizing activity as their expression in the overlying ectoderm is contemporaneous with this dorsalizing activity and they can mimic the effect of the overlying ectoderm (Liem et al., 1995).

Paired-box-containing genes (Pax genes) present a remarkable pattern of expression in the developing neural tube (for review: St-Onge et al., 1995). While transcripts of *Pax3* and *Pax7* are confined to the dorsal part of the neuroepithelium, *Pax6* is expressed most strongly in the ventral half excluding the floor plate region and its vicinity (Goulding et al., 1991; Jostes et al., 1991; Walther and Gruss, 1991). The regionalized expression of these genes precedes the onset of cellular differentiation suggesting that they may confer positional identity as neural precursors become specified along the dorsoventral axis of the neural tube. The region-specific expression of Pax genes appears to be regulated by dorsoventral signals emanating from the notochord, floor plate and overlying ectoderm (Goulding

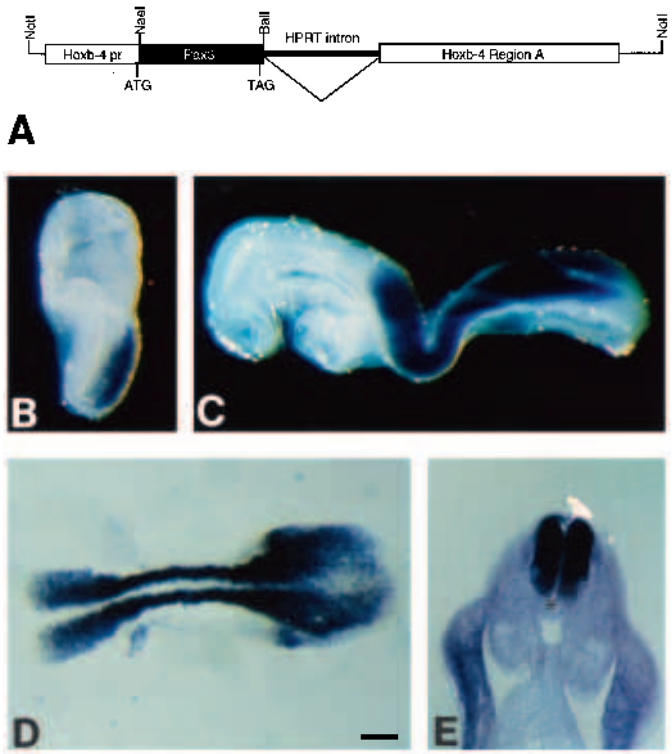

Fig. 1. Schematic diagram and early expression pattern of the *Hoxb-4A/Pax3* transgene. (A) Schematic presentation of the transgene utilized; the *Pax3* cDNA has been cloned between the *Hoxb-4* promoter and the *HPRT* intron/poly(A) sequence. The *Hoxb-4* region A enhancer is located downstream. (B-E) Ectopic *Pax3* expression of the *Hoxb-4A/Pax3* transgene at early embryonic stages as analyzed by whole-mount in situ hybridization. (B) *Pax3* is strongly misexpressed in the entire primitive streak of day 7.5-7.75 p.c. embryos. (C) Lateral view and (D) dorsal view of day 8.0 p.c. transgenic embryo presenting *Pax3* expression in the neural tube up to the rhombomere 6/7 boundary. Anterior is on the left and posterior on the right. (E) Vibratome section at the prospective hindlimb bud level of a normal transgenic day 9.5 p.c. embryo. Expression is detected in the entire neural tube but excludes the ventral midline (asterisk). (B-D) Bar, 250 μm; (E) bar, 100 μm.

et al., 1993a; Dietrich et al., 1993; Ang and Rossant, 1994; Liem et al., 1995). In the chick embryo, excision of the notochord rapidly results in the expansion of the *Pax3* expression domain to the most ventral region (Goulding et al., 1993a). In parallel, the differentiation of ventral cell types is inhibited as these notochordless neural tubes adopt a dorsalized fate. Inversely, grafting of a supernumerary notochord on the side of the developing neural tube represses *Pax3* expression in this region (Goulding et al., 1993a) and induces the ectopic differentiation of floor plate and motor neurons (van Straaten et al., 1985; 1988; Yamada et al., 1991). Similarly, mouse mutants lacking floor plate and/or notochord display *Pax3* expression extending to the ventral neuroepithelium (Dietrich et al., 1993; Ang and Rossant, 1994). Recent experiments performed on chick neural plate explants indicated that *Pax3* is also positively regulated by dorsalizing signals emanating from the overlying ectoderm (Liem et al., 1995). Altogether, these observations indicate that *Pax3* responds to dorsoventral signals patterning the neural tube and support the idea that *Pax3* acts as a regulator of cell specification within the neural tube.

The study of *Splotch* mutants, which harbor mutations in *Pax3* (Epstein et al., 1991, 1993; Goulding et al., 1993b), underlined the importance of *Pax3* function during development: animals homozygous for these mutations present a pronounced spina bifida, exencephaly, as well as neural crest and muscle deficiencies (Auerbach, 1954; Moase and Trasler, 1989; Franz, 1989, 1990; Bober et al., 1994; Goulding et al., 1994; Tremblay et al., 1995). Mutations in the human *PAX3* gene have also been linked to the human Waardenburg syndrome (Tassabehji et al., 1992; Morell et al., 1992) and cause craniofacial malformations as well as pigmentation deficiencies (for a review: Tremblay and Gruss, 1994).

The aim of this study was to determine whether *Pax3* can affect cell specification taking place along the dorsoventral axis of the neural tube. To answer this question, we used a *Hoxb-4*-derived expression vector (Whiting et al., 1991) to generate transgenic mice expressing *Pax3* in the entire neural tube at an early stage of development. In two transgenic lines harboring the *Hoxb-4A/Pax3* construct, we observed a pronounced phenotype in the brain as well as in the spinal cord. Analysis

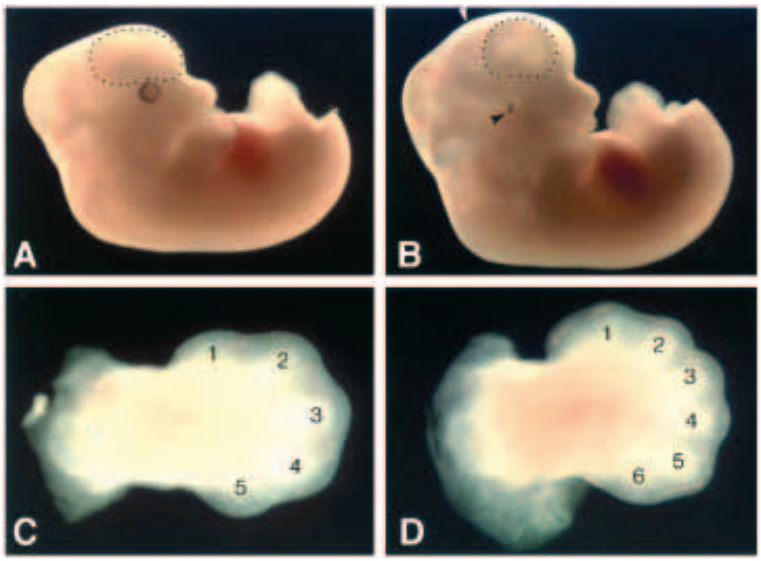

Fig. 2. Phenotype of transgenic embryos at day 12.5 p.c. (A,C) Wild-type embryo. (B,D) Transgenic embryo presenting typical phenotypic features. (A,B) The shape of the telencephalic vesicles is abnormal (compare the region delineated by dots) and bulging of the brain is visible between the diencephalon and mesencephalon (white arrowhead). Microphtalmia is evident (black arrowhead). Developing hand plate of (C) normal embryo and (D) abnormal transgenic embryo presenting polydactyly (compare the digits as numbered).

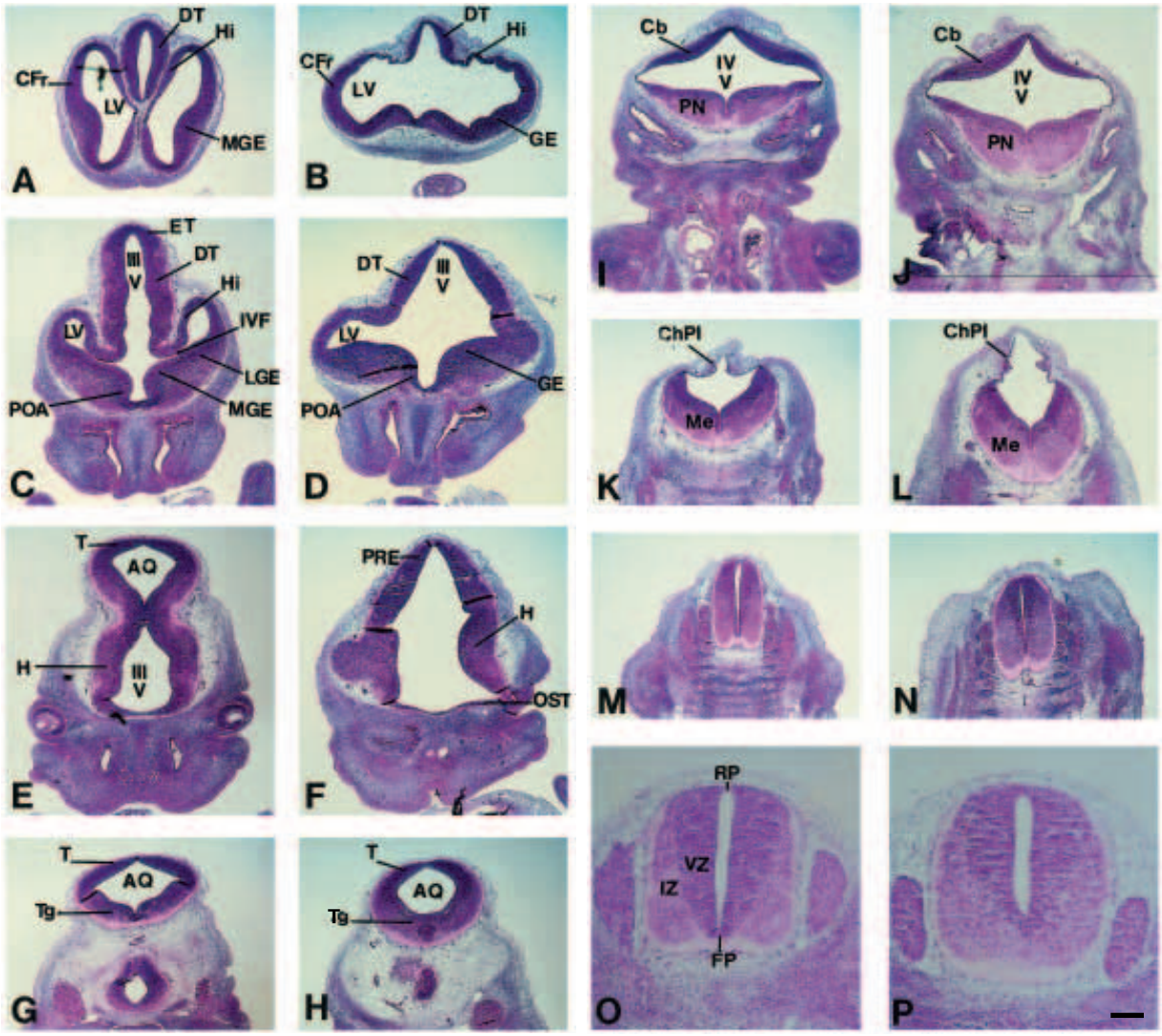

Fig. 3. Histological analysis of the brain and spinal cord of the affected transgenic embryos. Coronal sections through a day 12.5 p.c. wild-type embryo (A,C,E,G,I,K,M,O) and abnormal transgenic embryo (B,D,F,H,J,L,N,P). In the phenotypically affected embryo, note the aberrant shape of forebrain vesicles and their inability to separate from each other (A-F), the widened intraventricular foramen (C,D), the important dysgenesis of the telencephalic cortex, hippocampus (A,B) and dorsal thalamus (C,D), the abnormal optic stalk, lens and retina (E,F), and the thickened tegmentum together with the absence of ventral sulcus in the mesencephalon (G,H). The rhombencephalon (I-K, J-L) and the cervical spinal cord (M,N) do not present histological defects and differences in the thickness of the floor of these specimens are due to the angle of the sections. At the lumbar level (O,P), while the dorsal part of the spinal cord appears normal, the ventral part is abnormal. The ventricular zone is still identifiable but the distinctive wedge-shaped floor plate is unrecognizable in the abnormal embryo. An important accumulation of cells is noted at the ventral midline. AQ, aqueduct of Sylvius; Cb, cerebellar anlage; CFr, frontal cortex; ChPl, choroid plexus; DT, dorsal thalamus; ET, epithalamus; FP, floor plate; H, hypothalamus; Hi, hippocampus; IVF, interventricular foramen of Monroe; IZ, intermediate zone; LGE, lateral ganglionic eminence; LV, lateral ventricle; MGE, medial ganglionic eminence; Me, metencephalon; OST, optic stalk; POA, preoptic area; PN, pons; PRE, pretectal area; RP, roof plate; T, tectum; Tg, tegmentum; VZ, ventricular zone; III V; third ventricle; IV V, fourth ventricle. (A-N) Bar, 160 μm; (O,P) bar, 80 μm.

of the phenotype shows that *Pax3* is not sufficient to confer a dorsalized fate to cells differentiating in the ventral spinal cord. However, expression of *Pax3* ventrally inhibits the differentiation of the floor plate as analyzed using various molecular markers. This observation suggests that inhibitory events intrinsic to the neural tube play an important role in regulating floor plate differentiation. These data also provide a functional explanation for the rapid repression of *Pax3* in the neural tube prior to the differentiation of ventral structures and support the idea that Pax genes function as regulators of the dorsoventral patterning in the neural tube.

MATERIALS AND METHODS

Expression construct

A 1.6 kbp *Nae*I-*Bal*I fragment was purified from the *Pax3* cDNA (Goulding et al., 1991) and inserted in frame into the *Nco*I site (blunt

ended using Klenow) of p2.6A (kindly provided by R. Krumlauf; Whiting et al., 1991), a plasmid containing the *Hoxb-4* (formerly *Hox2.6*) promoter, the *HPRT* intron, the SV40 polyadenylation signal and the region A enhancer element of *Hoxb-4* to obtain the *Hoxb-4A/Pax3* construct (Fig. 1). The first 15 bp of the *Pax3* coding region were truncated while the start codon was provided by the p2.6A vector. The 7.5 kbp transgene was excised from the vector by a *Not*I digest, separated by agarose gel electrophoresis and gel purified using gelase (Epicentre Technologies).

Production of transgenic mice

Transgenic lines were produced by microinjection of (NMRI×B6D2) F_1 embryos as already described (Hogan et al., 1994). Eight transgenic animals (F_0) were obtained and bred with C57Bl/6J animals (Jackson Laboratory, Bar Harbor) to establish transgenic lines (designated T6-1 to T6-8). Some animals obtained from this breeding (F_1) were also mated to SJL, BALB/c, C3H, 129/Sv and DBA/2 mice to verify the effect of different genetic backgrounds. Mice were genotyped by Southern blot using a *Pax3 Kpn*I-*Kpn*I fragment (position 567-1504 on the cDNA; Goulding et al., 1991) labeled by random priming (Stratagene). Genomic DNA was prepared from the tail of 3-week-old animals as previously described (Hogan et al., 1994) and digested with *Pst*I (generating a single internal fragment) or with *Hin*dIII (generating two internal fragments as well as a distinctive flanking fragment) to confirm the origin of the embryos analyzed. Embryos were collected at various stages considering the morning when the vaginal plug was detected as day 0.5 p.c.

PCR analysis

Embryos were sometimes typed by PCR analysis using genomic DNA isolated from yolk sacs or tails. Individual yolk sacs were digested overnight at 55°C in 500 μl of 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS in the presence of 500 μg/ml of proteinase K. The samples were extracted with phenol:chloroform, isopropanol, precipitated, centrifuged at 15,000 revs/minute for 20 minutes and dissolved in 50 μl TE. 1-2 μl were used for the PCR reaction using primers specific to the *Hoxb-4* promoter region and the 5′ region of the *Pax3* cDNA (*Hoxb-4*: 5′-GTT CAC TTG ACA GTA AGT AGG AG-3′; *Pax3*: 5′-GTT TGC TGC CGC CGA TGG CAC-3′). PCR conditions were as follows: denaturation: 95°C, 3 minutes; annealing: 58°C, 1.5 minute; elongation: 72°C, 2 minutes; denaturation 94°C, 1 minute; 30 cycles; final annealing: 1.5 minutes; final elongation 10 minutes. Under these conditions, a specific 409 bp fragment was detected. In some cases, the genotype was confirmed by Southern blot as described above.

Histological analysis

Embryos were fixed overnight at 4°C in 4% paraformaldehyde/PBS, washed in PBS, dehydrated in ethanol/PBS series (30, 50, 75, 95, 100%, 1 hour each) treated with xylene for 2 hours and embedded in paraffin. Sections (8 μm) were used for hematoxylin/eosin staining, for in situ hybridization analysis or for immunohistochemistry.

In situ hybridization analysis

^{35}S-labelled antisense RNA probes used in this study were prepared as previously described: *Pax3* (Goulding et al., 1991), *Pax5* (Asano and Gruss, 1992), *Pax6* (Walther and Gruss, 1991), *Pax7* (Jostes et al., 1991), *Wnt-3a* (Roelink and Nusse, 1991), *Sax-1* (Schubert et al., 1995), *HNF3β* (Sasaki and Hogan, 1993), *Shh* (Echelard et al., 1993), *Msx-1* (probe 2, *BssHII* fragment; Hill et al., 1989), *Lhx-3* (Zhadanov et al., 1995) and *ChAT* (Ishii et al., 1990). The in situ hybridization analysis and the whole-mount in situ procedure were performed as previously described (Wilkinson 1992; Stoykova and Gruss, 1994). The *Pax3* probe did not distinguish between the endogenous and transgene expression but wild-type embryos were always tested in parallel to assess the normal *Pax3* expression pattern.

Immunohistochemistry

Immunohistochemistry was performed using two monoclonal antibodies: 40.2D6 recognizing Isl-1 and Isl-2 (Developmental Studies Hybridoma Bank), and NN18 directed against NF160 (Sigma). Sections were processed for immunostaining according to Karamitopoulou et al. (1994). Briefly, after xylene treatment and rehydration, sections were incubated for 30 minutes in 0.5% H_2O_2/methanol, placed in 10 mM citrate buffer (pH 6) and heated in the microwave at 750 watts for 5 minutes, 8 times. The sections were placed for 30 minutes in TBS (Tris-buffered saline, pH 7.6) and preblocked for 20 minutes in 10% heat-inactivated FCS/TBS. The primary antibody was applied at the appropriate dilution and incubated overnight at 4°C. After washes in TBS, the secondary antibody (Cy3-conjugated antimouse IgG, Jackson Immunoresearch) was applied for 1 hour at room temperature. Following washes in TBS, sections were mounted using Mowiol 4-88 (Hoechst) and observed using an axiophot epifluorescence microscope (Zeiss).

RESULTS

Production of transgenic mouse lines expressing *Pax3* ectopically

In order to elucidate the function of *Pax3* in the dorsoventral patterning of the neural tube, we introduced the *Pax3* cDNA into the p2.6A expression construct (Whiting et al., 1991; Fig. 1A). Gene expression driven by this vector construct has been shown to mimic the expression pattern of the *Hoxb-4* gene within the neural tube, from the hindbrain rhombomere 6/7 boundary to the caudal end of the axis (Whiting et al., 1991). A detailed description of this expression pattern has already been reported (Dickinson et al., 1994).

Using *Hoxb-4A/Pax3* construct, we generated 8 transgenic mouse lines overexpressing *Pax3* in the entire developing spinal cord. We first compared the transgene expression with the data obtained by others using the same vector (Whiting et al., 1991; Dickinson et al., 1994). Our data were in agreement with previous reports. While endogenous *Pax3* transcripts were not detected before day 8.5 p.c. of development (Goulding et al., 1991), we consistently detected strong ectopic expression of *Pax3* in the primitive streak of day 7.5-7.75 p.c. transgenic embryos (earliest stage analyzed; Fig. 1B). Similar analysis performed at day 8.0 p.c. (7 somites) revealed *Pax3* transcripts in the ventricular zone of the hindbrain from rhombomere 6/7 boundary and in the rostral neural tube. At these levels, *Pax3* expression was always excluded from the prospective floor plate area (hindbrain and cervical levels; Fig. 1C,D).

From day 9.5 p.c. the expression of the transgene adopted a different dorsoventral distribution according to the axial level analyzed, in agreement with the previous report (Dickinson et al., 1994). While in the hindbrain and the rostral part of the spinal cord (anterior to the forelimb) expression was weaker and excluded the floor plate area (data not shown), at more posterior levels the expression was stronger and included the entire neural tube (Fig. 1E) as well as, in the most caudal region, the prospective floor plate region (data not shown). At day 10.5 p.c., positive cells were found in the entire ventricular and intermediate zones but, by day 12.5 p.c., nearly all the expressing cells were located in the dorsal intermediate zone (Dickinson et al., 1994 and data not shown). Therefore, the ventral expression is likely to be repressed by ventral signals or down-regulated following cellular differentiation. However,

some transgenic embryos presented a pronounced brain and neural tube phenotype (see below). When tested, all these embryos presented a persistent *Pax3* expression all over the neural tube (*n*=5; Fig. 4).

In addition, a faint signal was detected around day 9.5 p.c. in the forelimb bud and in the body wall next to the prospective hindlimb bud area of the transgenic embryos (Fig. 1E and data not shown). This labeling from the transgene was distinct from the endogenous *Pax3* signal from the lateral dermomyotome (Bober et al., 1994) which was never detected in control embryos at the time when the reaction was terminated. At day 10.5 and 12.5 p.c., ectopic *Pax3* expression was also detected in the developing limbs (data not shown).

Ectopic expression of *Pax3* leads to developmental anomalies in two independent transgenic mouse lines

Lines T6-1 and T6-5 demonstrated the highest levels of *Pax3* expression together with line T6-8 (data not shown). Starting at day 10.5 p.c. we observed, in some embryos obtained from these two lines, a pronounced brain phenotype associated with multiple externally discernible defects (*n*=10; Fig. 2A-D): the shape of the telencephalic vesicles was abnormal and bulging of the brain was observed at the junction between the diencephalon and mesencephalon. In both lines, brain defects were associated with a wavy neural tube, microphtalmia and, occasionally, exencephaly or median facial cleft (Fig. 2B and data not shown). Polydactyly (6 or 7 digits) was always associated with this phenotype (*n*=8; Fig. 2D).

The penetrance of the phenotype was 6.8 and 11.5% for lines T6-1 and T6-5, respectively, when only embryos obtained from crossing F_1 animals were considered or 1.9 and 2.3% when embryos from all the consecutive crosses (F_2 and F_3) were included in the analysis (Table 1). Crosses performed using F_2 and F_3 animals did not produce abnormal transgenic embryos. Animals from lines T6-1 and T6-5 were also crossed for 1 to 2 generations to SJL, BALB/c, C3H, 129/Sv and DBA/2 animals to verify the effect of varying the genetic background on the phenotype. Embryos obtained from these crosses appeared normal. The reason for this low penetrance is not clear, but could be due to some genetic modifiers affecting the onset or level of transgene expression.

Since the defects observed in these embryos matched the regions of persistent ventral *Pax3* expression and were obtained in two independent transgenic lines as verified by Southern blot analysis (see Methods for details), we concluded that the phenotype was caused by ectopic expression of *Pax3*.

Brain and spinal cord defects associated with *Pax3* ectopic expression

Numerous histological anomalies were observed in the brain of every affected transgenic embryo examined (*n*=5). Dysgenesis of the telencephalic cortex was prominent and the hippocampal anlage failed to invaginate (Fig. 3B). The interventricular foramen was not properly formed resulting in lateral ventricles being largely opened on the third ventricle (Fig. 3D). The dorsal thalamus was abnormally shaped (Fig. 3D) and the pretectum extended more posteriorly than in a normal embryo (Fig. 3E-F). While these embryos appeared microphtalmic externally, histology revealed abnormally shaped optic stalks and vesicles with unrecognizable lens and retina (Figs 2B, 3F). The dorsal mesencephalon appeared normally shaped but the ventral medial sulcus was absent. The tegmentum was thicker showing an abnormal accumulation of cells in its ventral region (Fig. 3H). Both the metencephalon and myelencephalon appeared normal (Fig. 3I-N). Therefore, defects observed in the brain affected the telencephalic, diencephalic and mesencephalic areas.

Table 1. Phenotypic frequency as observed in two *Hoxb-4A/Pax3* transgenic mouse lines

	Hoxb-4A/Pax3 transgenic line analyzed	
	T6-1	T6-5
F_1 crosses:		
(F_1×F_1 or F_1×C57BL/6)	3/44 (6.8%)	6/52 (11.5%)*
Overall mating	3/162 (1.9%)	7/300 (2.3%)
Abnormal phenotypes		
Day 10.5 p.c.	1	1
Day 12.5 p.c.	2	5
Day 13.5 p.c.	0	1

Numbers are expressed as the number of phenotypically affected embryos over the total number of transgenic embryos examined. Phenotypic features scored were those externally observable: polydactyly, ocular and brain defects. These defects were always associated with neural tube defects as observed by histology.

*One phenotypically affected embryo is not presented in this category as it was obtained from an F_1×F_2 cross.

The dorsal ventricular zone of the spinal cord appeared always normal. However, the ventral part of the neural tube presented an abnormally rounded shape due to the important accumulation of cells in the ventral intermediate zone (*n*=7/7 examined; Fig. 3P). At high magnification the characteristic wedge-shaped floor plate was unrecognizable but motor neurons were still morphologically distinguishable in these areas (*n*=7/7 examined; Fig. 3P; high magnification not shown). These phenotypic changes were not observed along the entire axis as sections made at the hindbrain level (Fig. 3I-L) and the cervical level revealed a morphologically normal spinal cord (Fig. 3N; and data not shown). In contrast, the accumulation of cells in the ventral part of the spinal cord was striking at the forelimb level, although affected and unaffected regions were observed at this level. From the mid-thoracic level to the lumbar level of the developing neural tube, all sections presented the strong ventral phenotype described above. At the sacral and more caudal levels, the histology of the spinal cord appeared more normal as no cells had accumulated at the ventral midline (Fig. 5F,H).

In affected areas, the notochord was closer to the ventral part of the spinal cord and fewer sclerotomal cells appeared to have colonized this area (Fig. 5B-N). This phenotypic feature was clearly observable not only at the thoracic level (Fig. 5D,M) but also more caudally at the sacral level where no ventral cellular accumulation has been detected (Fig. 5H). As the floor plate appears to be missing in these regions (see below), this observation may reflect the importance of the floor plate not only in the induction of the sclerotome (Pourquie et al., 1993) but also in their guidance to constitute a normal vertebral body.

For this study, we focused our attention to phenotypically affected embryos obtained at day 12.5 and 13.5 p.c. and analyzed their CNS defects using molecular probes.

Abnormal patterning of the spinal cord revealed by molecular markers

In order to determine whether the ventral expression of *Pax3* is accompanied by a dorsalization of the neural tube, we analyzed the expression pattern of dorsal markers in the spinal cord: in day 12.5 p.c. control embryos, *Wnt-3a* (Parr et al., 1993) and *Msx1* (Hill et al., 1989; Robert et al., 1989) were restricted to the roof and, *Pax7*, to the dorsal part of the neural tube but excluded the roof plate (Jostes et al., 1991). In affected areas of abnormal embryos (n=4), while the expression of *Wnt-3a* (Fig. 4I) and *Msx1* (data not shown) remained circumscribed to the roof plate region, the ventral border of *Pax7* expression was consistently shifted ventrally relative to ventral limit of the neuroepithelium but was never completely ventralized (Fig. 4F). This observation was confirmed by measuring along the dorsoventral axis the percentage of the neuroepithelium occupied by the *Pax7* signal in a phenotypically affected and in an age-matched control embryo: the percentages were 70% (±3%; n=4) and 60% (±1; n=4), respectively, for four affected axial levels compared and 61% and 58% (n=1), respectively, at the unaffected cervical level.

The expression pattern of *Pax6* was analyzed in abnormal day 12.5 (n=3) and 13.5 p.c. (n=1) transgenic embryos (Fig. 4J-Q). In wild-type day 12.5 p.c. embryos, *Pax6* transcripts were detected in the ventricular zone of the spinal cord, mainly in the ventral part but clearly excluded the floor plate area and its vicinity (Fig. 4K). A weaker signal

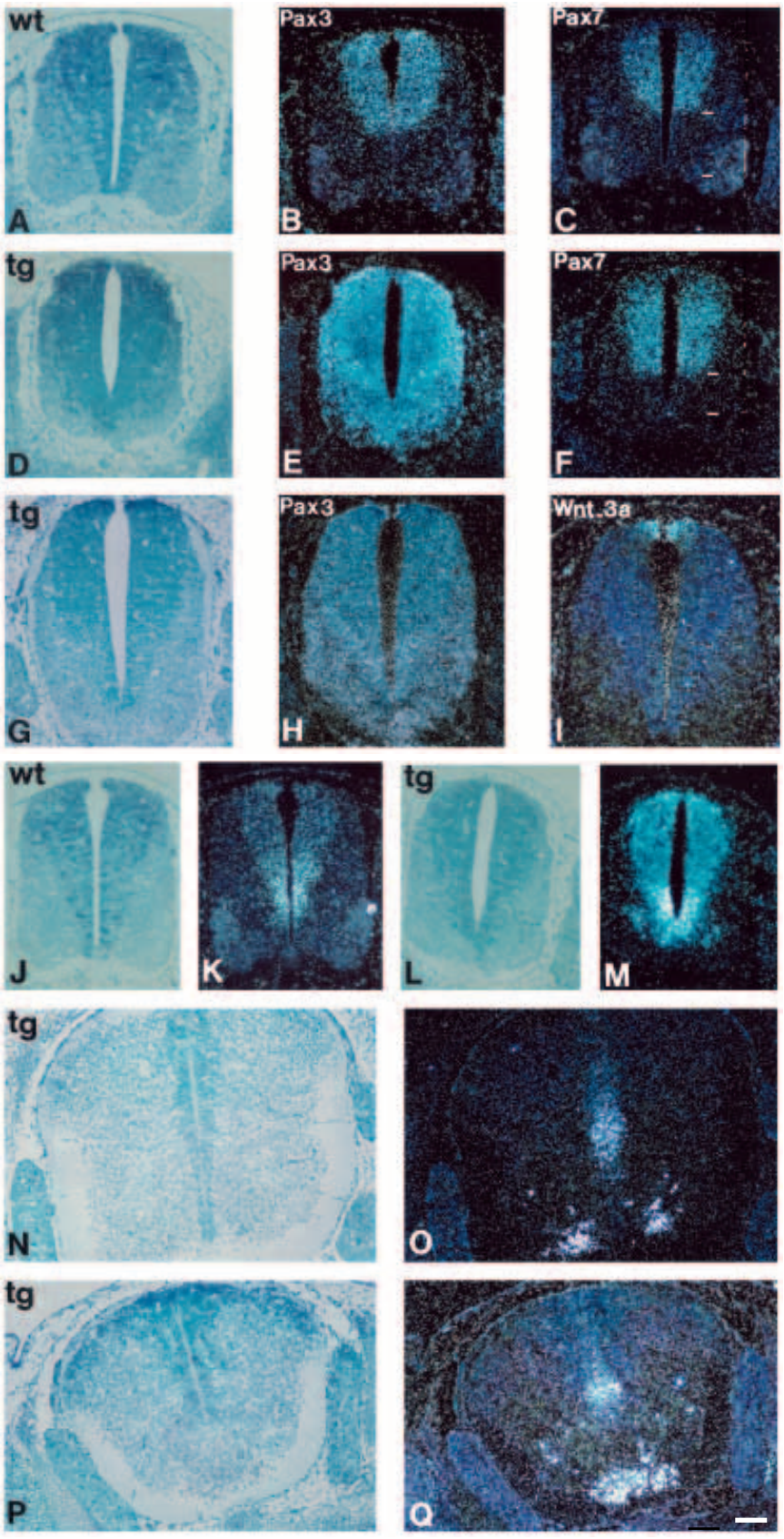

Fig. 4. Comparison of *Pax3*, *Pax7*, *Wnt-3a* and *Pax6* expression domains in wild-type and abnormal transgenic embryos at day 12.5 p.c. Cross sections showing the domain of *Pax3* expression in a wild-type (B) and in two abnormal transgenic embryos (E,H) at day 12.5 p.c. Note the strong expression of *Pax3* all over the spinal cord including the ventral midline. In abnormal embryos, the ventral border of *Pax7* expression is positioned more ventrally (in C and F compare the distance between the white bars corresponding to the ventral neuroepithelium where no *Pax7* is found) while the pattern of expression of the dorsal gene *Wnt-3a* is not modified (I). (A,D,G) Corresponding bright fields. In day 12.5 p.c. wild-type embryos *Pax6* transcripts are present in the ventricular zone of the spinal cord but exclude the ventral third (K). In the transgenic embryos, *Pax6* transcripts are detected in the most ventral part of the ventricular zone (M). (J,L) Corresponding bright fields. (N-Q) Affected embryo at day 13.5 p.c. present a normal distribution of *Pax6* transcripts in unaffected regions of the spinal cord: at the cervical level expression is detected in the ventricular zone and in two groups of cells flanking the floor plate area (O). In the affected areas, these two groups of *Pax6*-positive cells are fused at the ventral midline (Q). N,P, corresponding bright fields. Bar, 80 μm.

was detected in the dorsal part of the tube. (Fig. 4K; Walther and Gruss, 1991). A similar pattern was detected at day 13.5 p.c., although the ventricular expression domain was reduced as the mitotically active neuroepithelium regressed. In addition, some *Pax6*-expressing cells were visualized as they had migrated from the ventricular zone and settled bilaterally in the intermediate zone (data not shown; Walther and Gruss, 1991). The distribution of *Pax6* transcripts was unchanged in histologically normal sections taken at the cervical level of abnormal transgenic embryos (Fig. 4O). In phenotypically affected areas, two differences were observed. First, at day 12.5 p.c., *Pax6* transcripts were detected at the ventral midline of the ventricular zone, in the region where the floor plate normally differentiates (Fig. 4M). Second, at day 13.5 p.c., the two lateral columns of postmitotic *Pax6*-positive cells were fused in the intermediate zone at the ventral midline (Fig. 4Q).

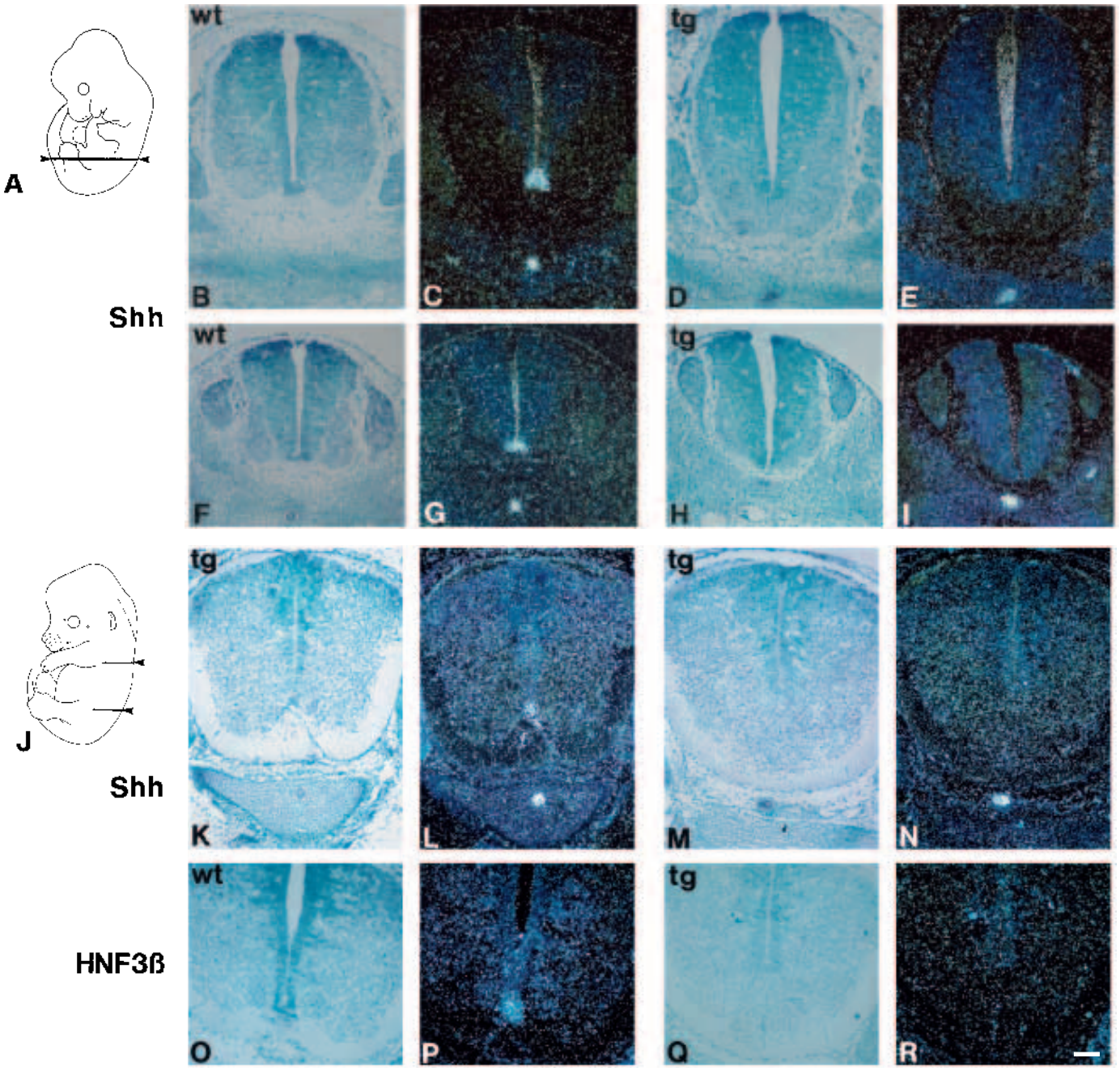

Fig. 5. In situ analysis of the expression of *Shh* and *HNF3β* transcripts in the wild-type and in the embryos misexpressing *Pax3*. Schematic representations of a day 12.5 p.c. (A) and of a day 13.5 p.c. (J) mouse embryo showing the level of the sections: (C,E) thoracic level (right arrow on A); (G,I) caudal level (left arrow on A); (L) cervical level; (N) thoracic level (top arrow on J); (P,R) lumbar level (lower arrow on J). *Shh* transcripts are present in the notochord of wild-type as well as transgenic embryos (C,E,G,I,L,N) but absent from the ventral midline of the neural tube specifically in morphologically affected areas of abnormal transgenic embryos (E,I,N). *HNF3β* is also absent at the ventral midline in the affected embryo (R) as compared to the wild-type embryo (P). Bar, 80 μm.

Expression of *Pax3* interferes with floor plate differentiation

The morphological anomalies observed, together with the expression of *Pax7* shifted ventrally and of *Pax6* occupying the ventral midline, prompted us to verify whether the floor plate differentiated in these embryos; we examined the expression of two floor plate markers, *Shh* and *HNF3β*, using in situ hybridization (Echelard et al., 1993; Sasaki and Hogan, 1993). In control day 12.5 and 13.5 p.c. embryos, transcripts of *Shh* were detected at the ventral midline of the neuroepithelium, from the mesencephalic level to the caudal part of spinal cord excluding the tip of the tail (Fig. 5C,G and data not shown). In addition, *Shh* labeled the notochord. In affected embryos (n=2), *Shh* was expressed along the entire notochord, but its expression was absent from the ventral midline of the spinal cord specifically in phenotypically affected regions (Fig. 5E,I,N). *Shh* transcripts were present in the floor plate at the cervical level (Fig. 5L) while no staining was detected at the thoracic (Fig. 5E,N), lumbar (data not shown) and sacral levels (Fig. 5I). Interestingly, at the sacral level of the spinal cord, although we did not observe an abnormal accumulation of ventral cells, *Shh* transcripts were always absent (Fig. 5I), suggesting that the absence of *Shh* (inhibition of floor plate differentiation) is an early event that precedes the ventral accumulation of cells. *HNF3β* expression analyzed along the spinal cord was consistent with *Shh* analysis (n=2; Fig. 5O-R). At day 13.5 p.c., *HNF3β* transcripts were detected in the floor plate area at the lumbar level of normal embryos (Fig. 5P) but not of affected embryos (Fig. 5R) where *Shh* was also absent. These data demonstrate that expression of *Pax3* can inhibit floor plate differentiation.

Neuronal differentiation in phenotypically affected transgenic embryos

We then assessed the status of neuronal differentiation in the spinal cord of the phenotypically affected embryos (n=2). We first performed immunocytochemistry using an anti-neurofilament antibody on control and phenotypically affected embryos at day 12.5 (data not shown) and 13.5 p.c. (Fig. 6A,B). As evidenced by neurofilament staining, neuronal differentiation took place all along the spinal cord of the affected transgenic embryos. However, the ventral commissure was not visualized (Fig. 6B). This difference was only observed in histologically affected areas of the spinal cord.

We refined our analysis by assessing motor neurons differentiation using two markers for postmitotic motor neurons: a pan motor neuron marker recognizing Isl-1 and Isl-2 LIM homeodomain proteins (Karlsson et al., 1990; Ericson et al., 1992; Tsuchida et al., 1994) and choline acetyl transferase (*ChAT*; Ishii et al., 1990). The pattern of expression of these molecules was analyzed in the spinal cord of day 13.5 p.c. transgenic embryos. Motor neurons were present all along the spinal cord of affected embryos, as indicated by morphological examination. However, while at the cervical level motor neurons appeared unaffected (data not shown), at the thoracic level, where the spinal cord morphology was strongly affected and *Shh* transcripts were absent from the neural tube, motor neuron columns were greatly decreased in size as evidenced by the reduced Isl-1/Isl-2 and *ChAT* labeling (Fig. 6C-D, E-F). Counting of Isl-1/Isl-2 immunoreactive cells revealed that the number of motor neurons in affected regions was reduced by 50% (mean number of motor neurons: control, 36.5 ±2.7 s.e.m.; transgenic, 18.0 ±1.7 s.e.m.; 8 and 16 motor columns counted respectively; P<0.05). Conversely, at the caudal level (sacral), the pattern of motor neuron markers remained unchanged (data not shown). Although this appears contradictory with the absence of *Shh* at the floor plate in this area, it is consistent with the normal histological appearance of the spinal cord at that level. Therefore, the number of motor neurons appeared severely reduced at the thoracic level but unchanged at cervical and sacral levels.

The differentiation status of other ventral cell types was assessed using *Lhx-3*, a LIM homeodomain gene (Zhadanov et al., 1995) and apparent homologue of *Lim3* (Tsuchida et al., 1994). *Lhx-3* transcripts are restricted to the ventral half of the spinal cord (Zhadanov et al., 1995). The precise nature of cells expressing *Lhx-3* is unclear but the location of some of these cells indicate that they may contribute to motor columns. In the affected as well as in the wild-type embryos, numerous *Lhx-3*-positive cells were visualized as they migrated away from the ventricular zone (Fig. 6G,H). However, in affected areas of transgenic embryos, their spatial distribution was different and the median columns were difficult to visualize (Fig. 6H). The *Lhx-3*-negative domain, including part of the lateral columns (Fig. 6G) was reduced or absent in the affected spinal cord (Fig.6H), indicating that some neuronal populations located in the ventrolateral region were reduced or absent. These data suggest that, while the induction and/or the differentiation of motor neurons appeared strongly hindered in morphologically affected areas of the spinal cord, the differentiation of other ventral cell types took place despite their abnormal final position, probably resulting from the absence of floor plate and of some motor neuron populations.

Pax3 affects the dorsoventral patterning of the mesencephalon

The mesencephalon of affected embryos also presented a histologically recognizable phenotype: the medial ventral sulcus was absent while a cellular accumulation was found in the ventral region (tegmentum). In order to assess whether the situation in the mesencephalon parallels the observations made in the spinal cord, we examined the dorsoventral patterning of the mesencephalon by characterizing the expression pattern of *Pax3, Pax7, Pax5, Pax6* and *Shh.* At day 12.5 p.c., ectopic expression of *Pax3* was undetectable in the mesencephalic area. While the expression of the dorsal *Pax7* gene was unaffected (Fig. 7C,I), *Pax5* and *Pax6*, which normally label two stripes on both sides of the ventral midline, were shifted ventrally, occupying the ventral midline (Fig. 7D,J,E,K). Furthermore, *Shh* was never expressed at the ventral mesencephalic midline of affected embryos (Fig. 7L) in contrast to the mesencephalic area of normal embryos (Fig. 7F). Therefore, the *Pax3* transgene appears to induce changes in the mesencephalon similar to those identified in the spinal cord: abnormal accumulation of cells ventrally and absence of floor plate differentiation.

DISCUSSION

In order to investigate the function of the dorsally restricted *Pax3* gene in cell fate determination, we generated transgenic

mice ectopically expressing *Pax3* in the ventral part of the neural tube using the *Hoxb-4A* promoter/enhancer construct. Using two transgenic mouse lines, we show that *Pax3* expression is not sufficient to confer a dorsal fate to neural tube precursors located in the ventral neural tube but that it inhibits floor plate differentiation. In these conditions, motor neurons still differentiated although in fewer numbers. Detailed analysis confirmed that the phenotypically affected areas correlated with domains where *Pax3* transcripts were most abundant and found to occupy early the presumptive floor plate area, as previously documented (Whiting et al., 1991; Dickinson et al., 1994; this report). Finally, these transgenic embryos presented a brain phenotype resembling the human holoprosencephaly-polydactyly syndrome.

The penetrance of the phenotype was 6.8 and 11.5% for lines T6-1 and T6-5 respectively, when considering only first generation crosses (e.g. F_1×C57Bl/6) or I.9 and 2.3 % when considering overall crosses. The low penetrance is likely to be due to the onset and level of *Pax3* ectopic expression at a critical period of neural tube development since, in normal transgenic embryos, the ventral *Pax3* expression is rapidly repressed in response to ventralizing signals or during cellular differentiation. As the genetic background modifies the phenotype observed in transgenic mice (Sibilia and Wagner, 1995; Threadgill et al., 1995), the phenotypic outcome may further depend on genetic modifier(s) affecting the expression of the transgene or the ability of neuroepithelial midline cells to respond to ventralizing signals and differentiate into floor plate cells. The fact that the phenotype was solely observed when F_1 generation transgenics were mated underlines the importance of the genetic background in the development of this phenotype.

Expression of *Pax3* is not sufficient to dorsalize the developing neural tube

Pax3 expression was reported to be restricted to the tip of the neural folds prior to neural tube closure (Goulding et al., 1991). Upon neural tube closure, the *Pax3* expression domain expands down to the sulcus limitans delineating the dorsal from the ventral part of the tube. Animals harboring homozygous *Pax3* mutations (*Splotch* alleles) present spina bifida, exencephaly and multiple neural crest deficiencies indicating that *Pax3* is essential for the proper closure of the neural tube and the generation, migration and/or differentiation of neural crest cells (for a review: Tremblay and Gruss, 1994). *Pax3* expression has been shown to be downregulated by ventralizing signals prior to the differentiation of ventral cell types (Goulding et al., 1993a; Dietrich et al., 1993; Ang and Rossant, 1994; Liem et al., 1995) but also to be upregulated by dorsalizing signals (Liem et al., 1995). Taken together, these observations suggest that *Pax3* mediates dorsal functions and responds to both early ventralizing and dorsalizing signals.

Although the expression of the dorsal gene *Pax7* was partially shifted ventrally and *Pax6* expression was found ectopically at the presumptive floor plate area, we found that expression of *Pax3* in the ventral neural tube is not sufficient to induce the ectopic ventral expression of dorsal markers such as *Msx1*, *Wnt3a* or *Pax7*. The cells differentiating in the ventral intermediate zone of these transgenic embryos still expressed ventral markers (see discussion below) indicating that they had not acquired a dorsal fate. Therefore, our in vivo data suggest that, in the presence of the notochord, the ectopic expression of *Pax3* is not sufficient to confer a dorsal identity to cells differentiating in the ventral neural tube.

Pax3 inhibits floor plate differentiation

In affected regions, *Pax3* expression was maintained at the ventral midline and the floor plate was absent as observed morphologically and by the lack of expression of floor plate markers *Shh* and *HNF3β*. At the cervical level, *Pax3* expression was never found at the ventral midline, both *Shh* and *HNF3β* were expressed and the floor plate differentiated normally. Our data indicate that the maintenance of *Pax3* expression in the prospective floor plate area inhibits the ability of precursor cells to respond to ventralizing signals emanating from the notochord, thereby preventing their differentiation into a floor plate. These observations confirm previous studies performed in the chick embryo using notochord grafting experiments (Goulding et al., 1993a) and neural plate explants (Liem et al., 1995) suggesting that a rapid down-regulation of *Pax3* is necessary for the differentiation of ventral structures.

The absence of floor plate bears profound morphological consequences in the ventral part of the developing spinal cord as cells accumulated in the ventral intermediate zone. Interestingly, at the sacral level of day 12.5 p.c. embryos, this phenotype was undistinguishable, despite the absence of *Shh* at the ventral midline. This suggests that the inhibition of floor plate development is an early event that precedes and is not a consequence of the local cellular proliferation. Therefore, this specialized midline structure may be involved in local inhibition of cellular proliferation. In a phenotypically affected day 10.5 p.c. embryo (earliest stage at which the phenotype was distinguishable), the expression of *Pax6* was found over the prospective floor plate area from the affected thoracic area to the caudal end of the axis (data not shown). From our data at day 12.5 p.c., the expression of *Pax6* at the ventral midline appears to be incompatible with the presence of a floor plate as it always correlated with the absence of *Shh* expression at the ventral midline. Although we cannot completely exclude that the differentiation of some floor plate cells took place at an early stage and that these cells rapidly degenerated thereafter, our observations are consistent with the idea that the initiation of floor plate differentiation does not take place in these embryos.

The inhibition of floor plate differentiation may also result from the indirect effect of *Pax3* misexpression. In particular, *Pax6* transcripts found at the ventral midline may inhibit floor plate differentiation. Interestingly, the activin-induced repression of *Pax6* in neural plate explants is followed by the differentiation of an enlarged floor plate (Pituello et al., 1995). Furthermore, the ectopic floor plate differentiation induced by *Shh* in the neural tube of frog embryos is temporally and spatially restricted, reinforcing the idea that additional local signals confine floor plate differentiation (Ruiz i Altaba et al., 1995a). Therefore, *Pax3* may block floor plate differentiation either directly by modifying the ability of precursor cells to respond to ventralizing signals or indirectly by altering the expression of other floor plate regulators.

Absence of floor plate is compatible with the differentiation of ventral cell types

We used our transgenic embryos to analyse how the absence

of the floor plate affects the differentiation of ventral cell types. The ventral commissure was absent within the spinal cord regions deprived of a floor plate in agreement with in vitro experiments where the floor plate has been shown to attract spinal commissural axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990). Motor neurons were present all along the spinal cord but their number was reduced by 50% in morphologically affected areas (Isl-1/Isl-2-positive cells). These motor neurons underwent differentiation since they expressed a neurofilament marker (NF160) as well as *ChAT*. *Lhx-3*-expressing cells were always numerous. Finally, the bilateral groups of *Pax6*-expressing cells were fused under the ventral midline, in contrast to neuronal populations detected with other markers. The exact nature of these Pax6-expressing cells remains to be determined. However, as they do not appear to express Isl-1/Isl-2, they probably do not correspond to motor neurons. Therefore, the ventral expression of *Pax3* appears to be insufficient to change the differentiation fate of many ventral populations, despite the absence of the floor plate, of the ventral commissure and the clear reduction of motor neuron columns (detected by Islet antibody and *ChAT* in situ hybridization). The ventralizing factors released by the notochord are likely to compensate partially for the absence of the floor plate and induce the differentiation of motor neurons. Studies have already shown that *Shh* induces motor neurons in vitro even in the absence of floor plate cells (Tanabe et al., 1995).

Pax3 induces a brain phenotype resembling the human holoprosencephaly-polydactyly syndrome

In phenotypically affected embryos, a pronounced phenotype was also observed in the forebrain and midbrain areas. Analysis of the brain phenotype using various regional brain markers (*Shh*, *Pax6*, *Wnt3a*, *Sax1*, *Pax3*, *Pax7* and *Pax5*) indicated that the general dorsoventral patterning was strongly affected as the expression of floor plate marker *Shh* was missing at the ventral midline in the mesencephalon. At day 8.5, 10.5, 12.5 and 13.5 p.c., we found no ectopic expression of *Pax3* within the brain of these embryos. However, expression analysis performed at day 7.5-7.75 p.c. revealed a strong ectopic signal in the node and the primitive streak areas. Fate mapping studies performed at day 7.5 p.c. demonstrated that the node region contributes to the formation of ventral midline structures: while its ventral part contributes to the head process, giving rise to the anterior part of the notochord up to the midbrain level, its dorsal part contributes to anterior floor plate cells (Beddington, 1994; Sulik et al., 1994; Smith et al., 1994). In the chick embryo, the behavior of these prospective anterior floor plate cells and their rapid differentiation suggest that they are exposed to early inductive signals, possibly of planar origin, originating from Hensen's node (Schoenwolf et al., 1990; Ruiz i Altaba et al., 1995b). The expression of *Pax3* in the region of the node may affect the early signaling events in the primitive streak and interfere with the differentiation of the floor plate. We therefore

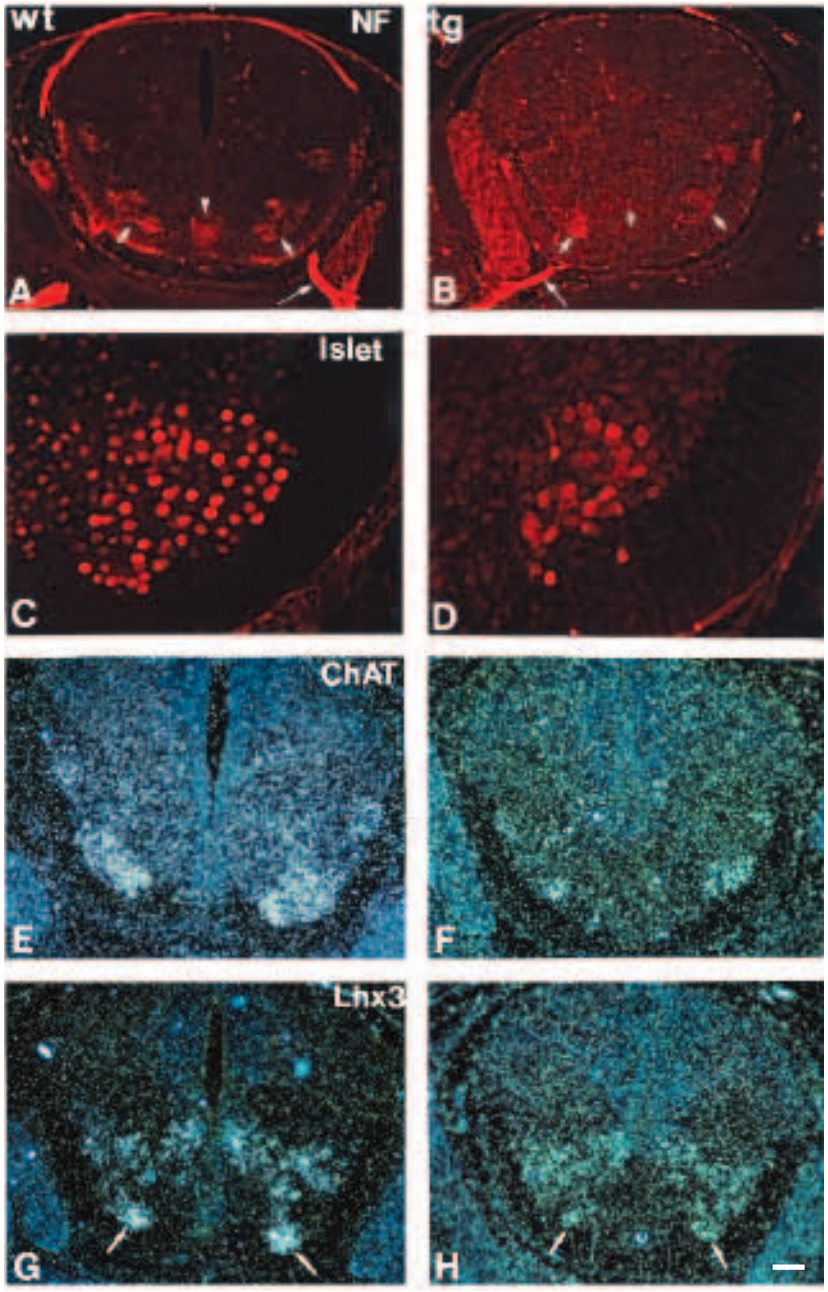

Fig. 6. Analysis of the ventral cell types differentiating in the spinal cord of abnormal embryos misexpressing *Pax3*. Cross sections of day 13.5 p.c. wild-type (A,C,E,G) and transgenic embryos (B,D,F,H). (A,B) Neurofilament immunoreactivity. The neurofilament is detected within the motor neuron columns (small arrow), in the ventral root (long arrow) but the ventral commissure (arrowhead) is not visualized in the affected embryo (B). (C,D) Islet-1/2 immunostaining and (E,F) ChAT in situ hybridization analysis showing that fewer motor neurons differentiate in the affected region of the spinal cord (D,F). (G,H) Numerous *Lhx-3* cells are produced in wild-type and transgenic embryos but the median *Lhx-3*-positive columns are not clearly identifiable in the transgenic embryos (arrow). (A,B) Bar, 100 μm; (C,D bar, 25 μm; (E,H) bar, 80 μm.

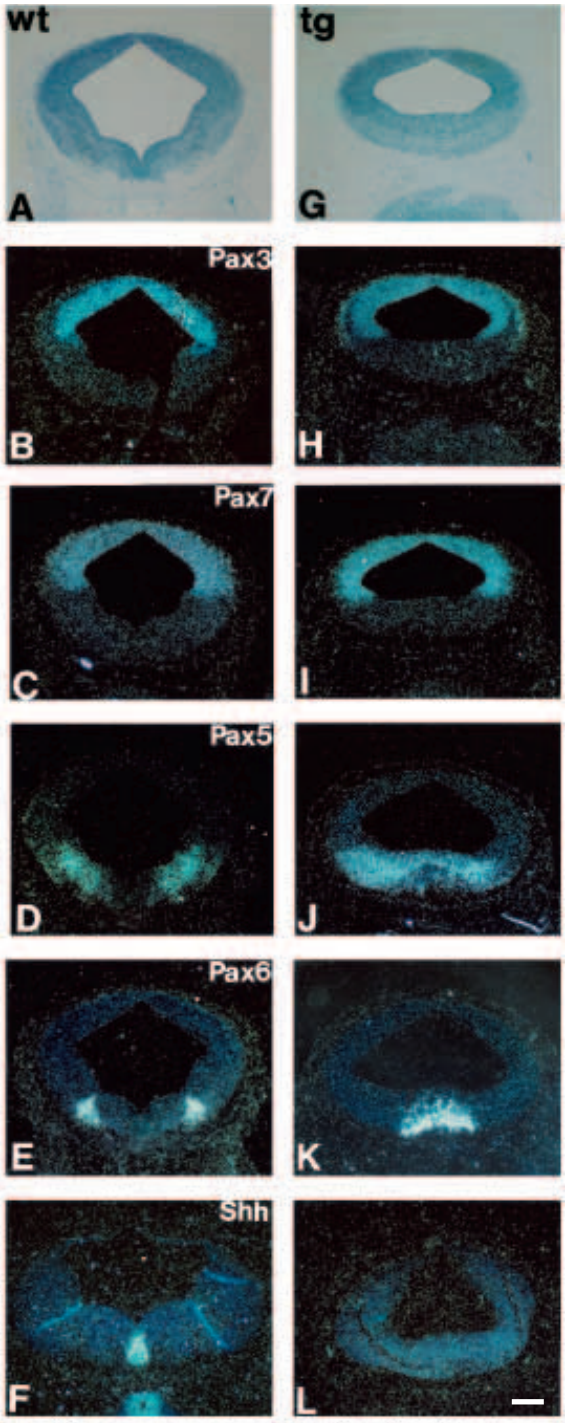

Fig. 7. Dorsoventral patterning in the mesencephalon. Cross sections through the mesencephalon of a day 12.5 p.c. wild-type (A-F) or of an affected embryo misexpressing *Pax3* (G-L). While the dorsal genes *Pax3* (B,H) and *Pax7* (C,I) are not affected, *Pax5* (D,J) and *Pax6* (E,K) present ectopic expression at the ventral midline. (F,L) The floor plate marker *Shh* is completely absent from the ventral midline. Bar, 160 μm.

suggest that the brain defects observed in our embryos result from midline anomalies due to an early misexpression of *Pax3* in the primitive streak.

Holoprosencephaly constitutes a genetically and etiologically heterogenous group of disorders and has been defined as a midline cerebral anomaly as the anatomical relationship between the two cerebral hemispheres is perturbed and the telencephalic vesicles fail to properly divide (for a review see Leech and Shuman, 1986). Craniofacial, visceral and musculoskeletal anomalies as well as polydactlyly are commonly associated with this malformation (Leech and Shuman, 1986, Lurie and Wulfsberg, 1993). Therefore, the forebrain, ocular and facial defects as well as the polydactyly observed in *Hoxb-4A/Pax3* embryos overlap with the pathological features of human holoprosencephaly. Our observations suggest that the development of human holoprosencephaly may be linked to mutations of midline regulators affecting the patterning of the forebrain possibly by regulating the early expression of *PAX3* and/or *PAX6.*

As more convenient promoters become available to target expression of Pax genes to the early primitive streak and to various domains of the spinal cord and brain, the establishment of new transgenic mice will provide interesting models to study the dorsoventral patterning and/or segmentation processes taking place within the spinal cord and the brain.

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