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Mouse *Wnt* genes exhibit discrete domains of expression in the early embryonic CNS and limb buds

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SUMMARY

Mutation and expression studies have implicated the *Wnt* gene family in early developmental decision making in vertebrates and flies. In a detailed comparative analysis, we have used in situ hybridization of 8.0- to 9.5-day mouse embryos to characterize expression of all ten published *Wnt* genes in the central nervous system (CNS) and limb buds. Seven of the family members show restricted expression patterns in the brain. At least three genes (*Wnt*-3, *Wnt*-3a, and *Wnt*-7b) exhibit sharp boundaries of expression in the forebrain that may predict subdivisions of the region later in development. In the spinal cord, *Wnt*-1, *Wnt*-3, and *Wnt*-3a are expressed dorsally, *Wnt*-5a, *Wnt*-7a, and *Wnt*-7b more ventrally, and *Wnt*-4 both dorsally and in the floor plate. In the forelimb primordia, *Wnt*-3, *Wnt*-4, *Wnt*-6 and *Wnt*-7b are expressed fairly uniformly throughout the limb ectoderm. *Wnt*-5a RNA is distributed in a proximal to distal gradient through the limb mesenchyme and ectoderm. Along the limb's dorsal-ventral axis, *Wnt*-5a is expressed in the ventral ectoderm and *Wnt*-7a in the dorsal ectoderm. We discuss the significance of these patterns of restricted and partially overlapping domains of expression with respect to the putative function of *Wnt* signalling in early CNS and limb development.

Key words: *Wnt* genes, CNS development, limb buds

INTRODUCTION

Normal development of vertebrate embryos requires the coordinate organization of groups of cells. Signalling between cells appears to be mediated, in part, by polypeptide growth factors and their receptors. A variety of studies have implicated members of the transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF) families in germ layer formation and axis determination during early embryogenesis (reviewed by Jessell and Melton, 1992). The *Wnt* gene family encodes a third group of cell signalling molecules that are likely to play important roles in vertebrate development (reviewed by McMahon, 1992; Nusse and Varmus, 1992).

A number of vertebrate *Wnt* genes have been cloned in recent years (see McMahon, 1992; Nusse and Varmus, 1992). *Wnt*-1 (*int*-1), the most intensively studied member of the family, was originally isolated as an proto-oncogene responsible for mammary tumors induced by mouse mammary tumor virus (Nusse and Varmus, 1982). *Wnt*-1 is normally expressed during early neural development in all vertebrates studied to date (Shackleford and Varmus, 1987; Wilkinson et al., 1987; Noordemeer et al., 1989; Molven et al., 1991; Bally-Cuif et al., 1992; McGrew et al., 1992). Mutational studies suggest a possible role for *Wnt*-1 in pattern formation during embryogenesis. Null alleles of *Wnt*-1, generated by homologous recombination in embryonic stem cells, result in the loss of midbrain and cerebellar structures in mouse embryos (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; McMahon et al., 1992).

The *Drosophila* homologue of *Wnt*-1 is the segment polarity gene *wingless* (Rijsewijk et al., 1987). *Wingless* is required for normal patterning in each segment of the *Drosophila* embryo (Nusslein-Volhard and Wieschaus, 1980; Baker, 1988), as well as correct development of the gut, imaginal discs, and Malpighian tubules (Sharma and Chopra, 1976; Baker, 1988; Cohen, 1990; Immergluck et al., 1990; Reuter et al., 1990; Skaer et al., 1992; Couso et al., 1993; Struhl and Basler, 1993).

Antibody staining of wingless protein has demonstrated its secretion and uptake by neighboring cells and restricted diffusion over a distance of a few cell diameters (van den Heuvel et al., 1989; Gonzalez et al., 1991). The mammalian Wnt-1 and Wnt-2 glycoproteins are also secreted and appear to act over short distances, as they are bound by the extracellular matrix and/or cell surface molecules (Brown et al., 1987; Papkoff et al., 1987; Bradley and Brown, 1990; Papkoff and Schryver, 1990; Blasband et al., 1992; Mason et al., 1992). In transgenic mice, overproliferation and/or tissue hyperplasia following the ectopic expression of *Wnt*-1 in the mammary glands, limbs, or spinal cord indicates that

the protein does possess growth factor-like activity (Tsukamoto et al., 1988; Zakany and Duboule, 1993; M. Dickinson, R. Krumlauf, and A. McMahon, unpublished data). To date, little is known about possible receptors for the Wnt proteins.

Given the known roles of *Wnt*-1 and *wingless* in embryogenesis, it is reasonable to expect that other *Wnt* genes also have important developmental functions. Experiments in *Xenopus* suggest that *Wnt* genes may be involved in primary axis formation during gastrulation. Injection of *Wnt*-1 or *Xwnt*-8 RNA into frog eggs or blastomeres can induce the formation of a secondary axis, including head structures (McMahon and Moon, 1989; Christian et al., 1991; Smith and Harland, 1991; Sokol et al., 1991). Neither *Wnt*-1 nor *Xwnt*-8 is normally expressed at the time when the primary axis is being established (Noordermeer et al., 1989; Christian et al., 1991), so that the ectopically expressed proteins may be activating the receptor for another *Wnt* family member (McMahon and Moon, 1989).

In situ hybridization studies also suggest that vertebrate *Wnt* genes are likely to have important roles in development. Four zebrafish *Wnt* genes are expressed in the brain, spinal cord, tail bud, and head mesoderm (Krauss et al., 1992). *Xenopus Xwnt*-4 is expressed in the floor plate of the spinal cord and dorsal regions of the brain (McGrew et al., 1992). *Xwnt*-7a RNA is present in the ventral neural tube and *Xwnt*-10 RNA in the dorsal hindbrain (Wolda and Moon, 1992). In mice, *Wnt*-5a RNA is distributed in a proximal-distal gradient along the limb buds of 9.5 day embryos and in a tissue-independent, regional domain at the caudal end of the gastrulating embryo (Gavin et al., 1990). The major sites of *Wnt*-3 and *Wnt*-3a expression during embryogenesis are along the dorsal midline of the brain and spinal cord (Roelink and Nusse, 1991; McMahon et al., 1992; Salinas and Nusse, 1992).

The expression of more than one *Wnt* gene in a given region raises the possibility of functional redundancy between different family members (McMahon and Bradley, 1990; McMahon et al., 1992). For example, *Wnt*-1 mutant embryos develop correctly in some areas, such as the spinal cord, where *Wnt*-1 is normally co-expressed with *Wnt*-3a (McMahon et al., 1992), suggesting that these genes may be functionally redundant. This conclusion is supported by *Xenopus* and mammary cell culture assays of *Wnt*-1 and *Wnt*-3a activity (Wolda et al., 1993; G. Wong, B. Gavin, and A. McMahon, unpublished data).

To provide a more complete picture of possible *Wnt* gene activities and the likelihood of functional redundancy between family members, we have performed a detailed comparative analysis of mouse *Wnt* gene expression during the initial development of the central nervous system (CNS) and limb primordia from 8.0 to 9.5 days of development. This period covers the initial induction and early patterning of these two structures and generally precedes the differentiation of specialized cell types. Thus, the CNS and limbs provide good examples of regions undergoing processes of pattern formation that might be guided by signalling factors like the Wnt proteins. The spatial and temporal patterns of *Wnt* gene expression suggest that the *Wnt* family may indeed play important roles in early CNS and limb development.

MATERIALS AND METHODS

In situ hybridization

(a) In situ hybridizations to paraffin sections were performed as described by Wilkinson et al. (1987) using single stranded ^{35}S-labelled RNA probes. 6 μm sections were cut and placed as two sets per microscope slide. This allowed the use of two different *Wnt* gene probes on each slide and a more accurate comparison of expression patterns. Photographs were taken on a Leitz Aristoplan microscope using Kodak Technical Pan 135 film.

Isolation of the cDNA and PCR clones used as in situ templates is described in Gavin et al. (1990). The *Wnt*-3a probe was provided by Drs R. Nusse and H. Roelink (Roelink and Nusse, 1991). The in situ probes listed below do not cross-react with each other: *Wnt*-1, 450 bp *Eco*RI-*Bam*HI (5′ untranslated and coding); *Wnt*-3, 380 bp PCR product (coding); *Wnt*-3a, 775 bp *Eco*RI-*Bgl*II (3′ untranslated); *Wnt*-4, 420 bp *Sma*I-*Sma*I (coding and 3′ untranslated); *Wnt*-5a, 360 bp PCR product (coding); *Wnt*-6, 336 bp *Eco*RI-*Xho*I (coding); *Wnt*-7a, 400 bp *Sma*I-*Eco*RI (coding and 3′ untranslated); *Wnt*-7b, 306 bp *Pst*I-*Eco*RI (3′ untranslated).

(b) Whole-mount in situ hybridizations were performed as in Wilkinson (1992) with the following modifications. Overnight incubation of embryos with hybridization mix was performed without rocking and with oil overlay. Pre-blocking of embryos with 10% sheep serum in TBST (140 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl pH 7.5, 0.1% Tween-20) was carried out for 3 hours. After the 30 minute, 70°C incubation of embryo powder in TBST, the solution was vortexed for 10 minutes prior to cooling on ice. After color developed to the desired extent, embryos were washed twice with NTMT (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.1% Tween-20) as indicated, but were then extensively washed with PBT (phosphate-buffered saline, 0.1% Tween-20) at pH 5.5 to prevent further alkaline phosphatase activity. These washes proved to be important for minimizing background staining, which might otherwise occur. Subsequently, embryos were post-fixed with 4% paraformaldehyde/0.1% glutaraldehyde in PBS (phosphate-buffered saline) for 1 hour, washed in PBT and stored at 4°C.

Digoxigenin-labelled RNA probes were prepared as in Wilkinson (1992). *Wnt* subclones used as probes were the same as for paraffin sections except for the *Wnt*-1 probe (1 kb *Sal*I-*Bgl*II; 3′ untranslated).

Photography was performed using an Olympus model SZH zoom stereo microscope with Kodak Ektachrome 160T professional slide film. Specimens were photographed in 80% glycerol in PBT.

RESULTS

Wnt genes exhibit discrete domains of expression in the embryonic brain

The embryonic vertebrate brain consists of three regions, the forebrain, midbrain, and hindbrain. As development proceeds from 8.0 to 9.5 days post coitum, the forebrain gives rise to two regions, the diencephalon and the anterior and laterally projecting vesicles of the telencephalon. Outpocketings from the diencephalon form the optic stalk and optic cup, which later generate the optic nerve and retina of the eye. The hindbrain also subdivides into two distinct regions, an anterior metencephalon, which gives rise, in part, to the cerebellum and a caudal segmented myelencephalon. We will describe patterns of *Wnt* gene expression with reference to these regions.

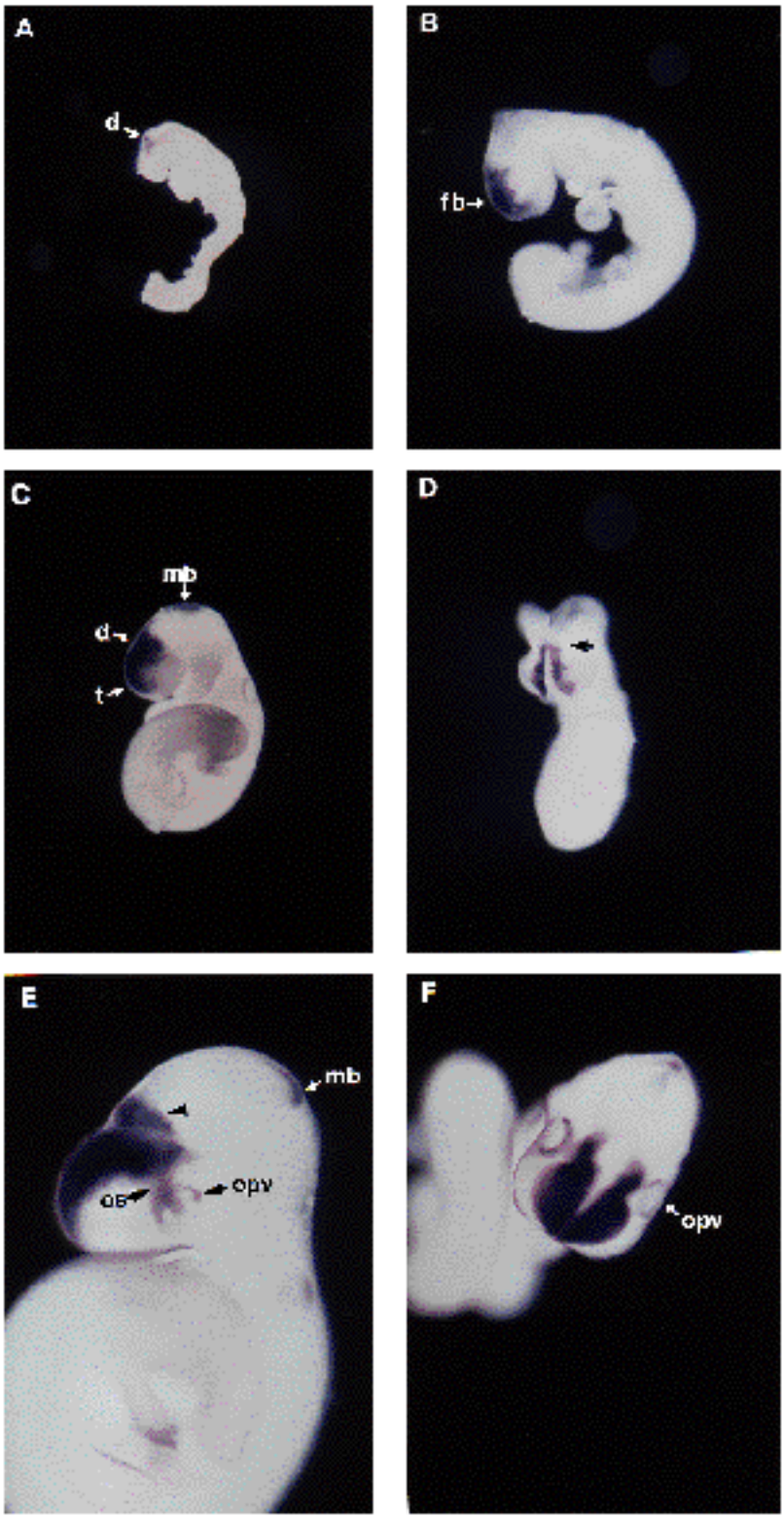


Fig. 1. Initial stages of *Wnt*-7b expression in the brain. (A) Whole-mount in situ hybridizations show *Wnt*-7b RNA in the presumptive diencephalon (d) at the 10 somite stage. (B) *Wnt*-7b expression then expands rostrally into the telencephalon to encompass much of the dorsal forebrain (fb) at the 13 somite stage. (C) By 9 days (17 somites), a faint band of *Wnt*-7b RNA is present in the midbrain (mb), in addition to strong telencephalic (t) and diencephalic (d) expression. (D) Frontal view of (B) demonstrates the sharp boundary (arrow) of *Wnt*-7b expression in the diencephalon. (E) At 9.5 days (24 somites), optic stalk (os), optic vesicle (opv), and caudal midbrain (mb) staining is evident. Interestingly, only the dorsal half of the optic vesicles express *Wnt*-7b. Forebrain expression shows varying intensities as a discrete block of weaker caudal diencephalon staining (arrowhead) contrasts with stronger rostral diencephalon and telencephalon expression. (F) Frontal view of (E) showing *Wnt*-7b forebrain expression including optic vesicle (opv) staining.

Forebrain

At 8.5 days of development (8-12 somites), the neural folds in the forebrain region are about to fuse. Subdivision of the region into the telencephalon and diencephalon is not yet apparent. The optic evaginations (vesicles) can be readily discerned by this period. Forebrain restricted expression of *Wnt*-7b and *Wnt*-3a is first detected between the 8 and 12 somite stages in the presumptive diencephalon, just anterior to the midbrain-forebrain junction (Figs 1A, 2A). Transcription of both genes is limited to a relatively small cluster of dorsal cells. Thus, expression of these two genes is one of the earliest markers of forebrain development.

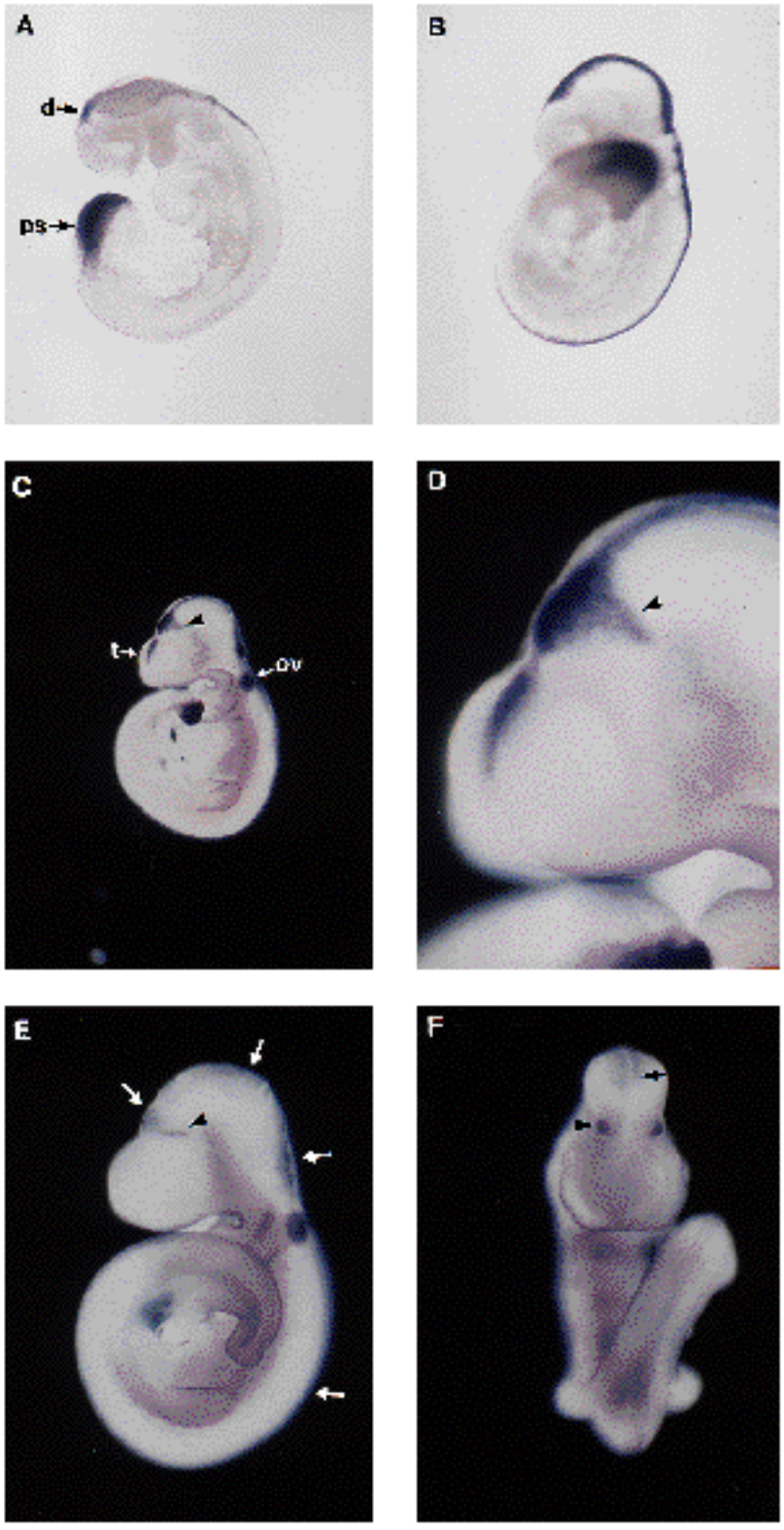


Fig. 2. Whole-mount in situ hybridizations of *Wnt*-3a and *Wnt*-3 expression in the brain from 8.5 to 9.5 days. (A) *Wnt*-3a expression in the diencephalon (d) in a 12-somite embryo. Expression in the primitive streak region (ps) is also evident. (B) At 9 days (17 somites), continuous dorsal midline expression of *Wnt*-3a extends from the forebrain through the spinal cord. (C) A sagittal view of *Wnt*-3a expression at 9.5 days (29 somites) shows dorsal midline staining extending into the telencephalon (t). *Wnt*-3a expression is also seen in a triangular patch in the lateral diencephalon (arrowhead) and the otic vesicle (ov). (D) A higher magnification view of *Wnt*-3a expression in the forebrain, including the triangular patch of staining in the diencephalon (arrowhead). (E) At 9.5 days (29 somites), weak *Wnt*-3 expression is seen along dorsal regions of the CNS (arrows) and laterally in the diencephalon (arrowhead). Note that this lateral extension of *Wnt*-3 expression is anterior to the *Wnt*-3a stripe in (C) and (D) and does not extend to the dorsal midline. At this time, there appears to be some limited overlap of *Wnt*-3 and *Wnt*-3a expression in the dorsal midline region of the diencephalon and possibly in the most ventral cells of the lateral stripe of *Wnt*-3 expression. (F) Frontal view showing the diencephalic patch of *Wnt*-3 staining (arrowhead). *Wnt*-3 expression is also evident at the dorsal midline up to the midbrain-diencephalon border (arrow). This dorsal midline expression extends in a spotty fashion through much of the diencephalon (see Figs 3, 5C).

From 8.5 to 9.5 days, forebrain development proceeds with the fusion of the neural folds, lateral extension of the optic stalks in the diencephalon, and rostral expansion of the telencephalic vesicles. Expansion of *Wnt*-7b expression from the diencephalon into a broad dorsal telencephalic domain, and *Wnt*-3a along the dorsal midline into the telencephalon accompanies the rapid growth and closure of the forebrain region (Figs 1B,C, 2B,C). A lateral stripe of *Wnt*-3a expression starts to emerge from the diencephalon at 9 days. By 9.5 days, this stripe has expanded into a triangular shaped wedge extending from the dorsal midline to the ventral diencephalon (Fig. 2C,D).

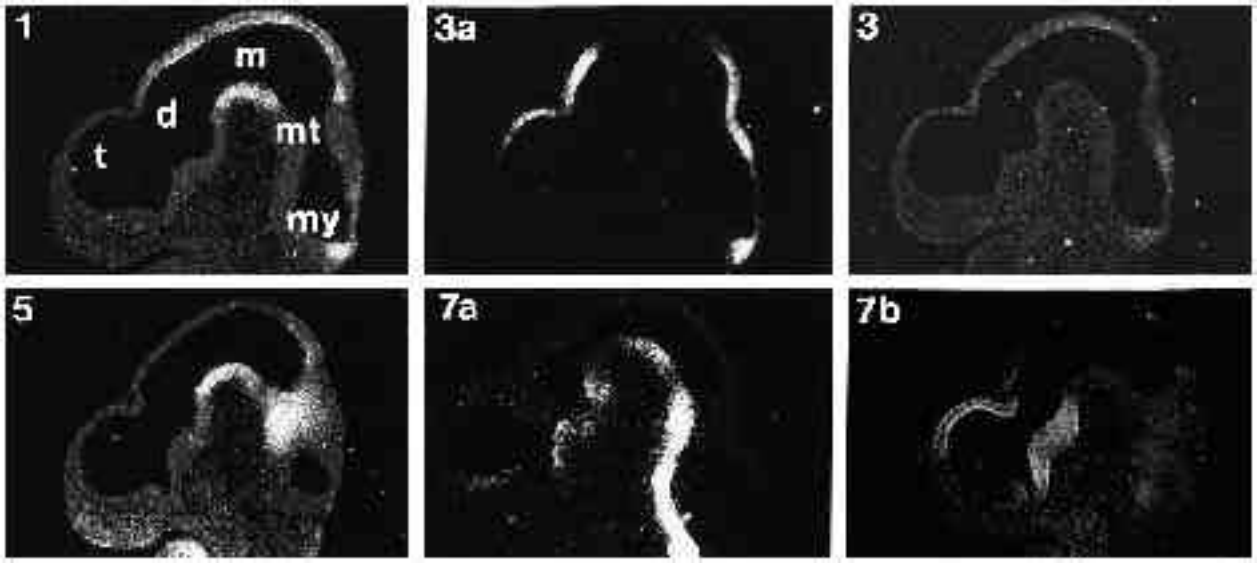


Fig. 3. Sagittal sections through a 9.5 day brain illustrate *Wnt*-1, *Wnt*-3a, *Wnt*-3, *Wnt*-5a, *Wnt*-7a, and *Wnt*-7b expression. Note that the dorsal *Wnt*-3a expression is continuous through the midbrain, but the plane of section is offset from the dorsal midline in this area. t, telencephalon; d, diencephalon; m, midbrain; mt, metencephalon; my, myelencephalon.

Wnt-3 RNA is first detected in the dorsal diencephalon around 9 days and soon appears in a lateral diencephalic stripe rostral and adjacent to the *Wnt*-3a stripe of expression (Fig. 2E,F). *Wnt*-3 is expressed in a patchwork fashion along the dorsal midline at this time, in contrast to the continuous midline expression of *Wnt*-3a.

By 9.5 days of development (23-30 somites), seven of the *Wnt* RNAs can be detected in the forebrain. Sagittal sections indicate that *Wnt*-1, *Wnt*-3, *Wnt*-3a, *Wnt*-4 (data not shown), and *Wnt*-7b are expressed primarily in the dorsal region, whereas *Wnt*-5a and *Wnt*-7a transcripts are mainly restricted to ventral areas (Fig. 3). *Wnt*-7b is also transcribed more ventrally in the diencephalon, including the optic stalk and the dorsal optic vesicle (Figs 1E, 3).

By using in situ hybridization to analyze a series of transverse sections through the 9.5 day brain (Fig. 4), we can clearly demonstrate the overlapping, yet unique, patterns of *Wnt* gene expression (Fig. 5). At the rostral end of the neural tube, *Wnt*-3a and 7b expression is evident in the telencephalon (Fig. 5A). *Wnt*-3a expression is confined to a small cluster of cells at the dorsal midline of the telencephalon, whereas *Wnt*-7b is strongly expressed throughout the dorsal and dorsolateral telencephalon.

In the rostral diencephalon, *Wnt*-3a and *Wnt*-7b are the only *Wnt* genes that are strongly expressed (Fig. 5B). Once again, *Wnt*-3a RNA is confined to dorsal midline cells, while *Wnt*-7b expression encompasses the entire dorsal half of the diencephalon and the dorsal portion of the optic vesicles. Although *Wnt*-7a transcription in the rostral diencephalon was not detected in these transverse sections, sagittal sections and whole-mount in situ hybridizations indicate that ventral *Wnt*-7a expression does extend this far anterior in the forebrain (Figs 3, 7F).

In the caudal half of the diencephalon, the domain of *Wnt*-3a expression is noticeably broader at the level of its dorsal to ventral stripe of expression (Fig. 1C,D), extending as far ventrally as *Wnt*-7b (Fig. 5C). *Wnt*-1 RNA can be detected at the dorsal midline (Fig. 5C). *Wnt*-3 is weakly expressed in the dorsal region, but more strongly in the narrow dorsal to ventral stripe previously mentioned (Figs 2E,F, 5C).

Wnt-5a expression is first detected at the 14 somites stage in the ventral half of the brain, where it abuts *Wnt*-7b

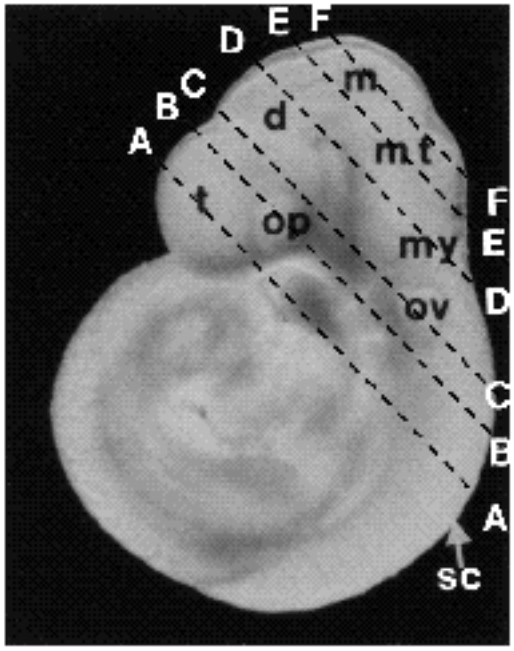


Fig. 4. Sagittal view of a 9.5 day mouse embryo. The lines (A-F) indicate the levels at which the corresponding transverse sections in Fig. 5 were cut. t, telencephalon; d, diencephalon; m, midbrain; mt, metencephalon; my, myelencephalon; sc, spinal cord; op, optic vesicle; ov, otic vesicle.

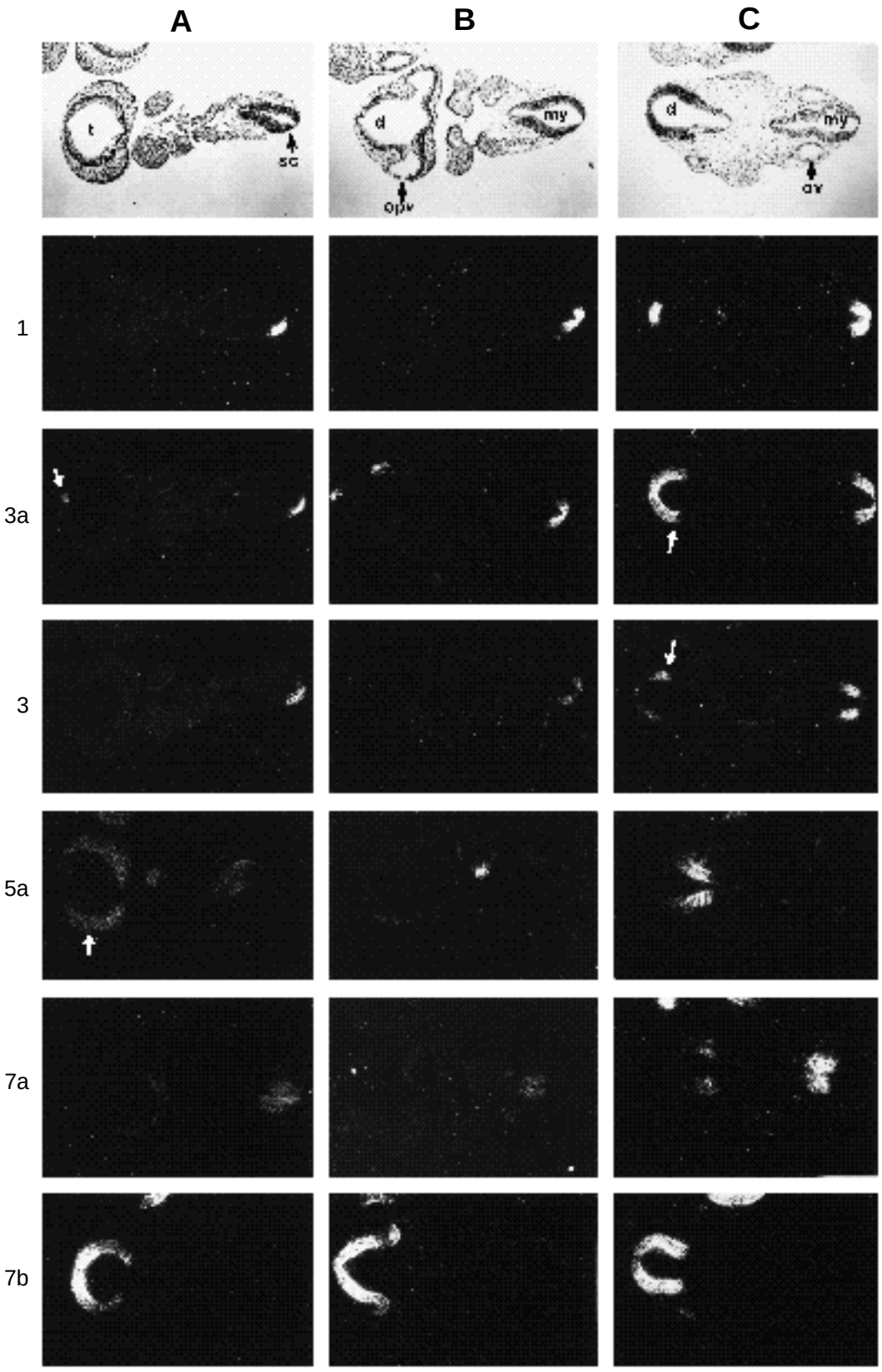
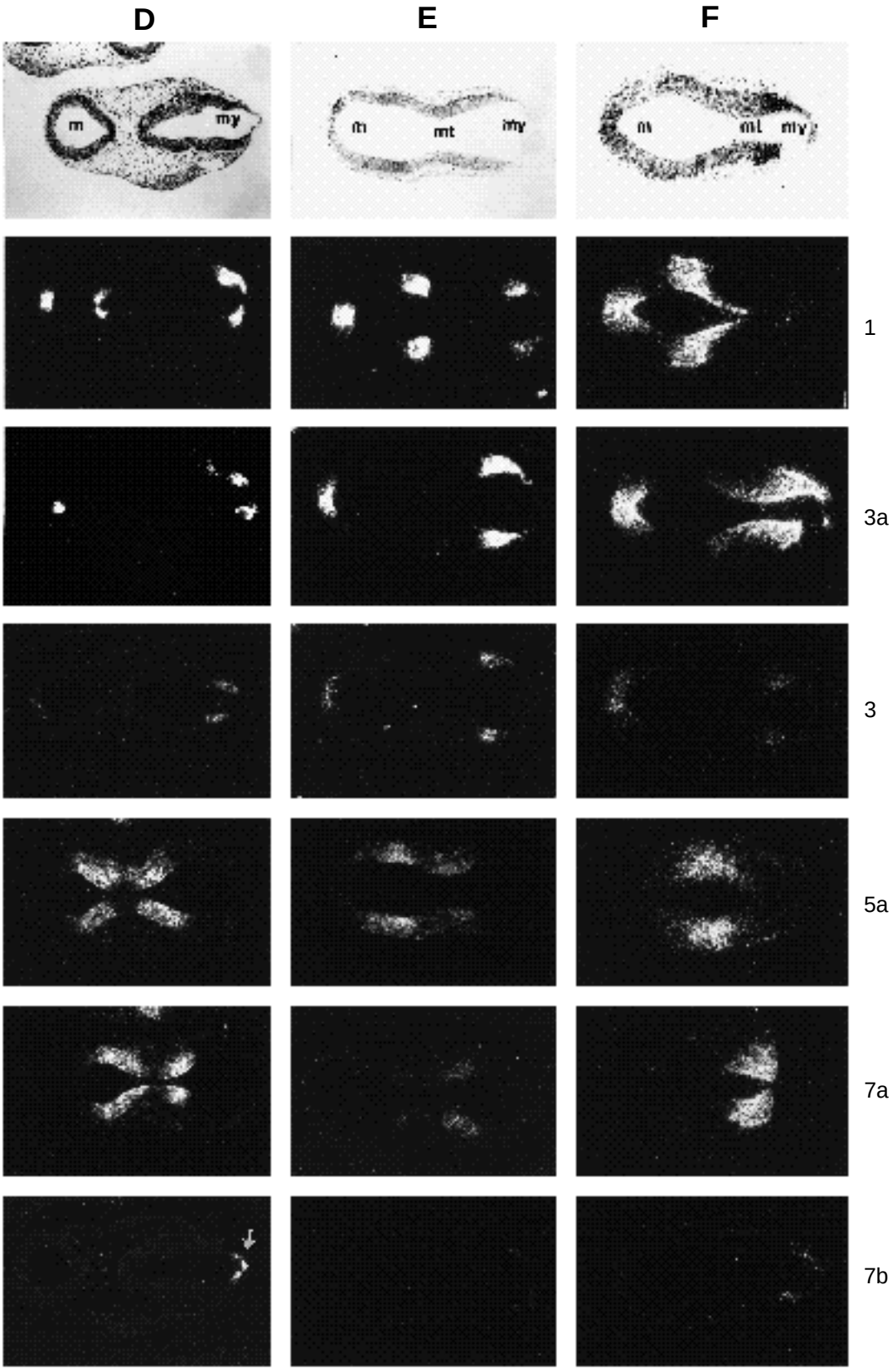


Fig. 5. Transverse sections through a 9.5 day brain show *Wnt* gene expression in the telencephalon (t), diencephalon (d), midbrain (m), metencephalon (mt), and myelencephalon (my), optic vesicle (opv), otic vesicle (ov), and spinal cord (sc). In A, the white arrows point out *Wnt*-3a expression at the dorsal midline of the telencephalon and *Wnt*-5a expression in facial neural crest. The white arrows in C



indicate the appearance of *Wnt*-3 expression in the diencephalon and the lateral extension of *Wnt*-3a expression. In C, the sections hybridized with *Wnt*-3 and *Wnt*-3a probes were not immediately adjacent to each other, so that the area of overlapping expression is mainly confined to the dorsal midline region. In D, *Wnt*-7b expression (arrow) is in the roof plate overlying the myelencephalon.

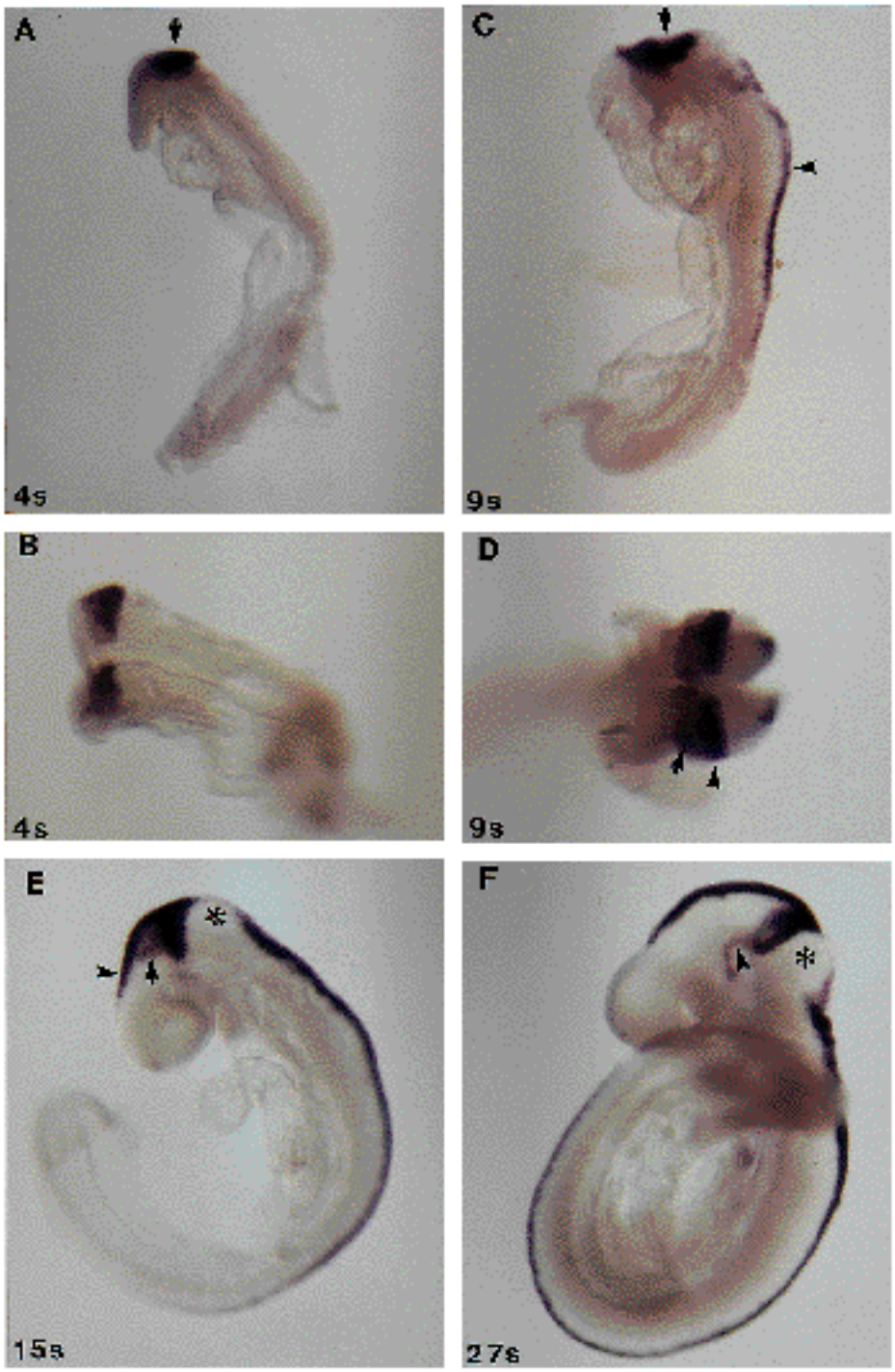


Fig. 6. Whole-mount in situ hybridizations of *Wnt*-1 expression in the CNS from 4 somite to 27 somite stages. (A) Sagittal view of *Wnt*-1 RNA in the midbrain at the 4 somites stage (arrow). (B) 4-somite embryo viewed from above illustrates the broad dorsal to ventral distribution of *Wnt*-1 RNA. (C) Sagittal view of a 9-somite embryo shows extensive *Wnt*-1 expression in the midbrain (arrow). Initial *Wnt*-1 expression in the spinal cord (arrowhead) can be seen. (D) 9-somite embryo viewed from above shows that anterior midbrain expression is decreasing (arrow), while more caudal expression remains strong (arrowhead). (E) At the 15 somites stage, strong dorsal midline *Wnt*-1 expression has extended into the diencephalon (arrowhead) and down much of the spinal cord. No dorsal expression is apparent in the metencephalon (asterisk). Extensive dorsal to ventral expression is still evident in the caudal midbrain, while ventral *Wnt*-1 expression is weak in the anterior midbrain (arrow). (F) The major site of *Wnt*-1 expression at the 27 somites stage is along the dorsal midline from the diencephalon through the spinal cord. However, no expression can be detected in the metencephalon (asterisk). All that remains of the once extensive *Wnt*-1 domain in the midbrain is strong dorsal midline expression, weak, transient expression just lateral to the ventral midline (arrowhead), and a circle of expression just anterior to the mid/hindbrain junction.

expression in the floor of the diencephalon (data not shown). At 9.5 days, *Wnt*-5a is strongly expressed in the ventral half of the caudal diencephalon, and *Wnt*-7a RNA is barely detectable in the same region (Figs 5C, 7F).

Although *Wnt*-4 RNA has been detected in the dorsal diencephalon and midbrain at 9.5 days (data not shown), the levels of expression appear to be extremely low. For this reason, it is difficult to be confident about exact spatial domains of expression; therefore, we will not discuss *Wnt*-4 in this context. In addition, we could not detect localized expression of *Wnt*-2, *Wnt*-5b, or *Wnt*-6 in the CNS (also see McMahon and McMahon, 1989; Gavin et al., 1990).

By 9.5 days, there are several striking examples of sharp boundaries delimiting the extent of *Wnt* gene expression in the forebrain. These boundaries may preview the segmental units (neuromeres) that are hypothesized to eventually subdivide the forebrain (see Puelles et al., 1987). Whole-mount in situ hybridizations highlight particularly the boundaries in the diencephalon for *Wnt*-3, *Wnt*-3a, and *Wnt*-7b. *Wnt*-3a and *Wnt*-7b share one of these boundaries in the dorsal-lateral region (Figs 1D-F, 2C,D), whereas the *Wnt*-

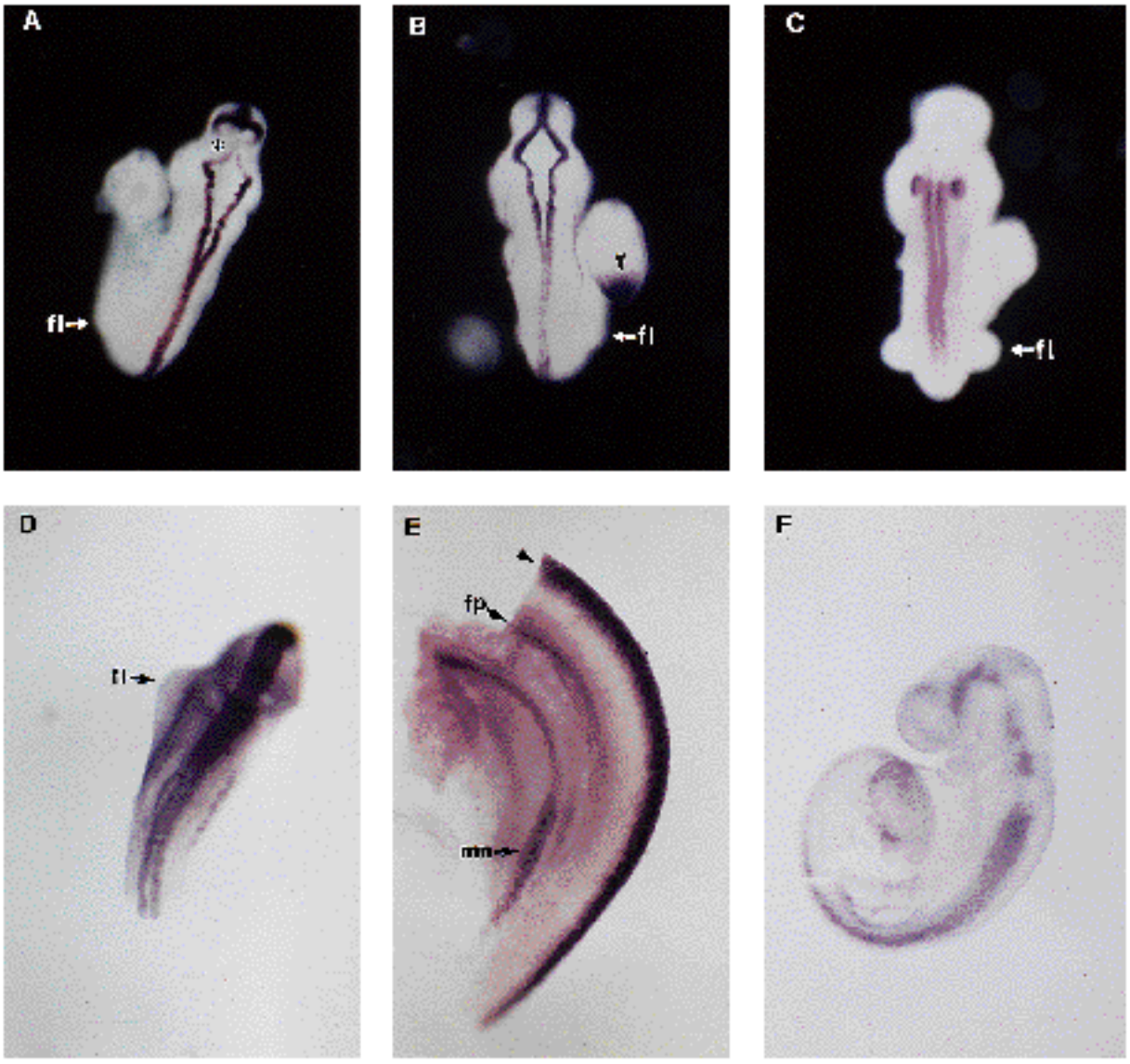


Fig. 7. Whole-mount in situ hybridizations of *Wnt* gene expression in the spinal cord at 9.5 days. (A) *Wnt*-1 expression in the dorsal midline of the hindbrain and spinal cord. Absence of *Wnt*-1 expression in the metencephalon is denoted by an asterisk. (B) *Wnt*-3a expression in the dorsal hindbrain and spinal cord. Expression in the tailbud is evident (arrowhead). (C) *Wnt*-3 expression in the spinal cord may be slightly offset from the dorsal midline and does not extend to the caudal limits of the cord. (D) Dorsal view of *Wnt*-4 expression in the spinal cord. (E) Sagittal view of *Wnt*-4 expression in the dorsal spinal cord (arrowhead), floor plate (fp), and mesonephric tubules (mn). (F) *Wnt*-7a expression in ventral regions of the brain and spinal cord. As a reference point, the level of the forelimbs (fl) is marked in panels (A-D).

3 boundary is more rostral (Fig. 2E,F). The resulting *Wnt*-3a and *Wnt*-3 'compartments' may correspond to the presumptive synencephalon and posterior parencephalon, respectively, of Puelles et al. (1987). Salinas and Nusse (1992) have noted the *Wnt*-3 restriction and shown that it demarcates the future dorsal thalamus. Transverse sections also reveal discrete dorsal-ventral boundaries in the expression of *Wnt*-1, *Wnt*-3, *Wnt*-3a, *Wnt*-5a, *Wnt*-7a, and *Wnt*-7b in the forebrain (Fig. 5A-C). Interestingly, it appears that the ventral boundaries of *Wnt*-3, *Wnt*-3a, and *Wnt*-7b expression may coincide with the dorsal boundaries of *Wnt*-5a and *Wnt*-7a expression. Thus the combined patterns of *Wnt* gene expression encircle the entire neural tube in this region.

Midbrain

Detailed examination of *Wnt*-1 expression in the midbrain has been reported previously (Wilkinson et al., 1987; McMahon et al., 1992). However, whole-mount in situ hybridization demonstrates a dynamic aspect to the expression of *Wnt*-1 not previously appreciated. *Wnt*-1 RNA is initially detected in the presumptive midbrain region at 8 days (one somite stage; McMahon et al., 1992). At the 4 somites stage, *Wnt*-1 RNA is clearly localized to the

midbrain (Fig. 6A) and occupies a broad dorsal to ventral distribution with the exception of the ventral midline (Fig. 6B). By the 9 somites stage, *Wnt*-1 shows extensive dorsal expression throughout much of the midbrain (Fig. 6C); however, rostral midbrain expression is diminished (Fig. 6D). By the 15 somites stage, neural tube closure is almost complete in the cranial region and strong *Wnt*-1 expression is now caudally restricted within the midbrain, continuing to occupy an extensive dorsal to ventral region just anterior to the mid/hindbrain junction (Fig. 6E). Anterior midbrain expression is weak in ventral regions, but strong at the dorsal midline where it extends into the diencephalon (Fig. 6E). By 9.5 days (27 somites), all that remains of the extensive *Wnt*-1 midbrain domain is a tight circle of expression (excluding the extreme ventral midline) just anterior of the mid/hindbrain junction and a dorsal midline stripe running from this circle forward through much of the diencephalon (Figs 3, 6F). There is also an area of ventral *Wnt*-1 expression throughout the midbrain and caudal diencephalon (Figs 3, 6F), which is not visible at other stages.

As noted previously (McMahon et al., 1992), *Wnt*-3a expression in the dorsal midbrain closely parallels *Wnt*-1 expression at this time (Fig. 5D-F). *Wnt*-3 transcripts remain confined to a faint band at the dorsal midline (Fig. 5D-F), which overlaps the domain of *Wnt*-1 and *Wnt*-3a expression. However, *Wnt*-3 expression is clearly much weaker. *Wnt*-5a and *Wnt*-7a are expressed in a remarkably similar broad region throughout the ventral and lateral midbrain, although the distribution of *Wnt*-7a RNA appears much more patchy (Figs 3, 5D-F). Interestingly, *Wnt*-7b expression is detected in a dorsolateral stripe near the midbrain-hindbrain junction (Fig. 1C,E; McMahon et al., 1992). However, unlike *Wnt*-1, this stripe does not extend ventrally.

Hindbrain

The initial expression and overlap of *Wnt*-1 and *Wnt*-3a in the dorsal hindbrain at 8.5 days has been reported previously (McMahon et al., 1992). At 9.5 days, *Wnt*-1 is not transcribed in the metencephalon, but is expressed dorsally in a continuous stripe from the rostral myelencephalon through the length of the spinal cord (Figs 3A, 5A, 6F; Wilkinson et al., 1987). In contrast, *Wnt*-3a RNA is present in the dorsal midline continuously from the telencephalon to the base of the spinal cord, including the metencephalon (Figs 2B,C, 5F). Thus, *Wnt*-1 and *Wnt*-3a RNAs are distributed very similarly in the hindbrain with the exception of the dorsal metencephalon. *Wnt*-3 RNA is present in the same dorsal regions of the 9.5 day hindbrain as *Wnt*-3a RNA but appears excluded from the extreme dorsal midline cells that express *Wnt*-1 and *Wnt*-3a (Figs 5B-F, 7C).

Wnt-7a is expressed ventrally and laterally throughout the hindbrain (Figs 3E, 5B-F, 7F), whereas *Wnt*-5a RNA is present in the ventral and lateral metencephalon but absent from most of the myelencephalon (Figs 3D, 5B-F; data not shown). *Wnt*-7b expression can be detected in a restricted area of the roof plate overlying the caudal myelencephalon (Fig. 5D). The ependymal layer is bulging outwards in this section of the roof plate and will eventually rupture to form the Foramen of Magendie (Nauta and Feirtag, 1986). This opening allows cerebrospinal fluid to pass from the fourth ventricle into the subarachnoid space surrounding the CNS.

Wnt genes display three patterns of dorsal-ventral restricted expression in the spinal cord

At 9.5 days, the mouse spinal cord consists primarily of an actively proliferating ventricular zone. The first spinal cord neurons are born at this time, and very little differentiation of distinct cell types has occurred (Nornes and Carry, 1978). In particular, there are no morphological indications of the prominent dorsal to ventral variations in neuronal cell types that will characterize the spinal cord later in embryogenesis. Despite this apparent uniformity in spinal cord morphology, patterns of *Wnt* gene expression demonstrate clear distinctions along the dorsal-ventral axis (Figs 7, 8). Three general patterns emerge and reflect a continuation of the expression profiles seen in the hindbrain. The first is exhibited by *Wnt*-1, *Wnt*-3, and *Wnt*-3a, which are expressed dorsally in the roof plate region (Figs 7A-C, 8). However, *Wnt*-3 expression does not extend into the most caudal portions of the spinal cord and may be down-regulated in extreme dorsal midline cells (Fig. 7C). *Wnt*-5a, *Wnt*-7a, and *Wnt*-7b exhibit a second pattern of expression in more ventral portions of the spinal cord (Figs 7F, 8; Gavin et al., 1990). None of these genes is expressed in the floor plate, however. *Wnt*-5a RNA is confined to the most rostral part of the spinal cord, as it is not found at the level of the forelimbs (Fig. 8; Gavin et al., 1990). *Wnt*-4 shows a unique transcription pattern (Figs 7D,E, 8). It is transcribed in a broad dorsal domain, which may exclude some roof plate cells, but encompasses the presumptive alar plate region. Moreover, *Wnt*-4 is the only known *Wnt* gene to be expressed in the floor plate at this time. *Wnt*-4 transcription in the floor plate of the spinal cord is also seen in *Xenopus* embryos (McGrew et al., 1992).

Wnt-5a and *Wnt*-7a are differentially expressed in the forelimb buds

The forelimb primordia first begin to protrude from the trunk region at 9.25 days of development. By 9.5 days, the expression of six *Wnt* genes can be detected in the developing limb bud (Fig. 8). *Wnt*-3, *Wnt*-4, *Wnt*-6, and *Wnt*-7b are expressed fairly uniformly throughout the limb ectoderm. In contrast, *Wnt*-7a is expressed only in the dorsal ectoderm of the limb. *Wnt*-7a RNA can be detected in the flanking ectoderm as the presumptive limb bud first begins to elongate (data not shown). Along the dorsal-ventral axis, *Wnt*-5a RNA is found primarily in the ventral half of the limb ectoderm (also see Gavin et al., 1990). In addition, *Wnt*-5a is the only known mouse *Wnt* gene to be expressed in the limb mesenchyme. As previously observed (Gavin et al., 1990), *Wnt*-5a is expressed in a proximal-distal gradient along the limb mesenchyme and ectoderm, with the highest level of expression seen in the distal ectoderm of the future apical ectodermal ridge and the underlying distal mesenchyme.

DISCUSSION

There are many striking features in the expression profiles of the mouse *Wnt* genes in the CNS and limb primordia. The timing of *Wnt* gene transcription often coincides with the determination of pattern or polarity, prior to overt differentiation of the tissues.

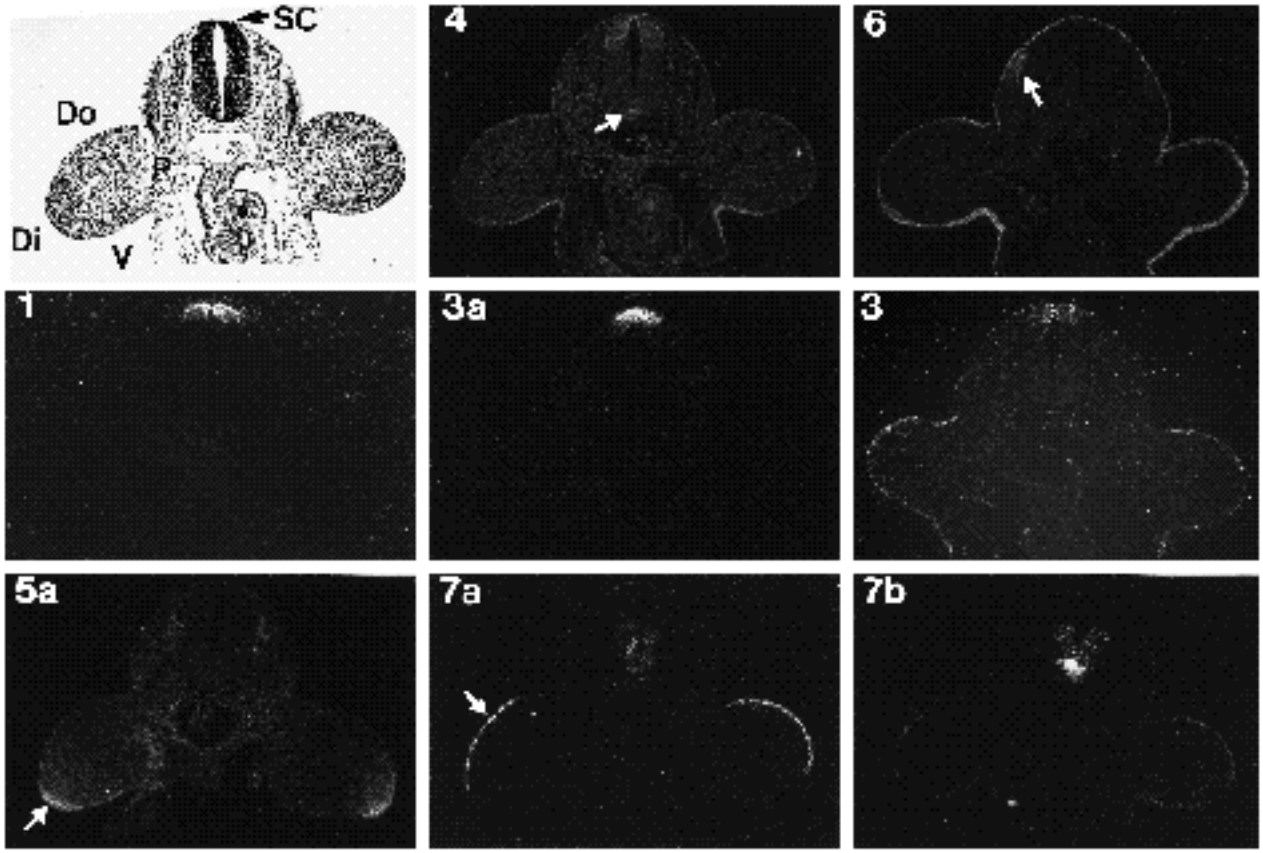


Fig. 8. A transverse section through a 9.5 day embryo at the level of the forelimbs illustrates the expression of *Wnt*-1, *Wnt*-3a, *Wnt*-3, *Wnt*-4, *Wnt*-5a, *Wnt*-6, *Wnt*-7a, and *Wnt*-7b in the spinal cord (sc) and limb buds. The arrows point to *Wnt*-4 expression in the floor plate of the spinal cord, *Wnt*-6 expression in the somites, *Wnt*-5a expression in the distal ectoderm of the limbs, and *Wnt*-7a expression in the dorsal ectoderm of the limbs. The limb bud axes are dorsal (Do), ventral (V), proximal (P), and distal (Di).

In discussing the significance of these expression patterns, it is crucial to recall that a given *Wnt* gene may have more than one function. Among the possible functions of *Wnt* genes are cell fate determination, proliferation, and cell survival. It is important to note that these activities are not mutually exclusive. For example, *wingless* can act as a determinant of cell fate choice in the segments of the *Drosophila* embryo and later function as a mitogen in Malpighian tubule cells (Nusslein-Volhard and Wieschaus, 1980; Skaer et al., 1992). Ectopic expression experiments in *Xenopus* indicate the potential for *Wnt* genes to affect cell fate choice during primary axis formation and mesoderm induction (Christian et al., 1991; Smith and Harland, 1991; Sokol et al., 1991). The ectopically expressed *Wnts* also create changes in gap junction permeability (Olson et al., 1991). In mice, ectopically expressed *Wnt*-1 has a mitogenic effect in mammary glands, limbs, and spinal cord (Tsukamoto et al., 1988; Zakany and Duboule, 1993; M. Dickinson, R. Krumlauf, and A. McMahon, unpublished data). *Wnt*-1 deficient mice, lacking regions of the midbrain and cerebellum, particularly illustrate the problems of distinguishing between the alternative roles that *Wnts* might play (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; McMahon et al., 1992). *Wnt*-1 expression in the midbrain may instruct the appropriate cells to become midbrain structures (cell fate or patterning). Alternatively, *Wnt*-1 could act as a mitogen or survival factor for midbrain cells. Similar hypotheses must be considered when evaluating *Wnt* gene function elsewhere in the developing embryo.

Wnt genes and CNS development

The primary region of mouse *Wnt* gene expression is the central nervous system (also see Wilkinson et al., 1987; Roelink and Nusse, 1991; McMahon et al., 1992; Salinas and Nusse, 1992). In the 8-8.5 day neural tube, *Wnt*-1, *Wnt*-3a, and *Wnt*-7b RNAs are initially detected around the time of neural fold closure in their respective domains of expression. By 9.5 days, expression of the various *Wnt* genes encircles much of the brain and spinal cord (summarized in Figs 9, 10).

These expression profiles suggest that *Wnt* signalling plays a major role in early CNS development. Gene targetting experiments have demonstrated that loss of *Wnt*-1 activity results in a deletion of midbrain and dorsal metencephalic structures (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; McMahon et al., 1992). However, *Wnt*-1 mutant mice exhibit no phenotypic abnormalities in the diencephalon, caudal hindbrain and spinal cord where *Wnt*-1 is normally expressed. It is possible that *Wnt*-3a, whose expression coincides with *Wnt*-1 in these areas, is function-

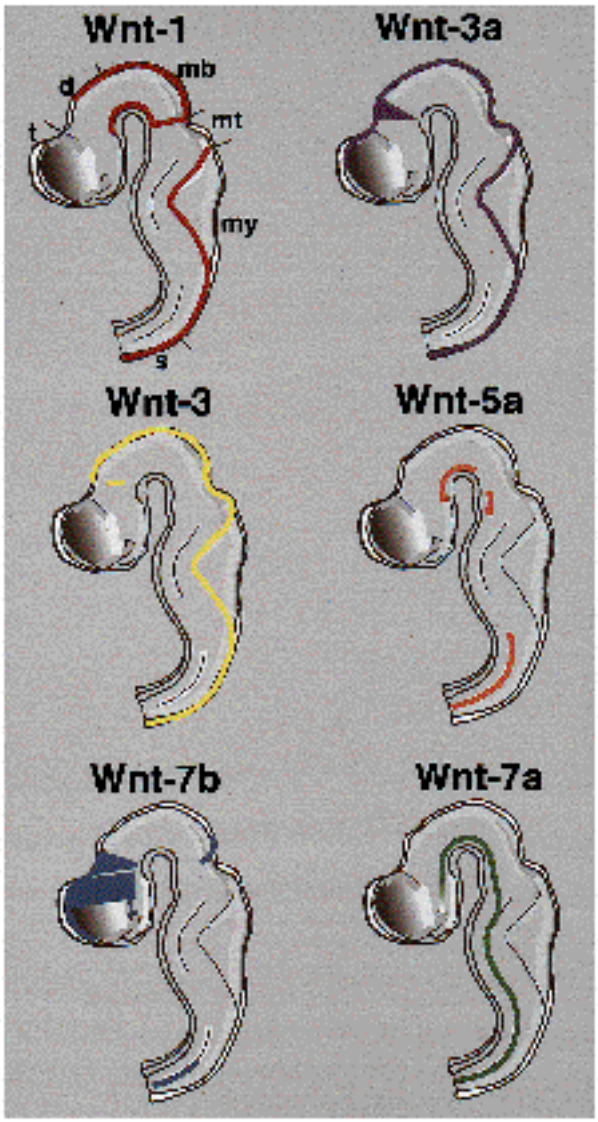


Fig. 9. Summary of *Wnt* gene expression in the neuroepithelium of the 9.5 day mouse brain and rostral spinal cord. t, telencephalon; d, diencephalon; mb, midbrain; mt, metencephalon; my, myelencephalon; s, spinal cord.

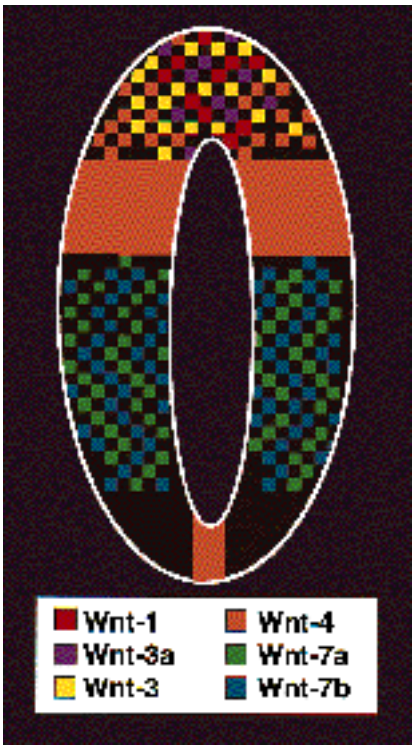


Fig. 10. Summary of *Wnt* gene expression in the 9.5 day mouse spinal cord. Dorsal is at the top, ventral at the bottom. Overlapping expression patterns are indicated by intermingled squares of different colors. This convention should not be interpreted as indicating expression of different *Wnt* genes in mutually exclusive populations of cells.

ally substituting for the loss of *Wnt*-1 activity (McMahon and Bradley, 1990; McMahon et al., 1992). Indeed, *Wnt*-1 and *Wnt*-3a appear to have identical effects when ectopically expressed in *Xenopus* embryos or mouse mammary epithelial C57MG cells (Wolda et al., 1993; G. Wong, B. Gavin, and A. McMahon, unpublished data).

The overlapping expression patterns of various *Wnt* genes in the brain and spinal cord indicate the potential for considerable functional redundancy during development. Does this mean that when different family members are expressed in the same region, they will perform essentially identical functions? Mammary cell transformation studies can separate *Wnt* activities into two classes: transforming (*Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3a, *Wnt*-7a, *Wnt*-7b) and non-transforming (*Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6) genes (G. Wong, B. Gavin, and A. McMahon, unpublished data), suggesting that there are at least two distinct signalling mechanisms/classes of receptors. Ectopic expression experiments in *Xenopus* further suggest that *Wnt*-4 and *Wnt-5a* also have activities that differ from each other as well as from *Wnt*-1 (McGrew et al., 1992; Wolda et al., 1993). Thus, even though there is a broadly overlapping distribution of *Wnt* signals, they may be performing quite distinct roles.

The existence of segmental units, or rhombomeres, within the vertebrate hindbrain has been well documented at the morphological and molecular levels (reviewed by Lumsden, 1990; Wilkinson and Krumlauf, 1990). There is also some morphological and histochemical evidence for segmentation in the forebrain (see Puelles et al., 1987). At 9.5 days of mouse development, there are no apparent morphological boundaries that would correspond to the forebrain neuromeres observed at later stages. However, as segmental units are initially established, one might expect patterns of gene expression to preview the morphological subdivisions. In fact, *Wnt*-3 and *Dlx*-1 are expressed in just such restricted domains in the diencephalon beginning at 9.5-10.5 days (Salinas and Nusse, 1992). Our results indicate that, in addition to the sharp boundaries of *Wnt*-3 expression, *Wnt*-3a and *Wnt*-7b expression in the forebrain at 9.5 days may prefigure the later forming neuromeres. Thus, *Wnt* genes may play a role in the specification of these putative developmental units.

In the spinal cord, *Wnt* gene expression is observed prior to or coincident with the birth of the first neurons at 9.5 days (Nornes and Carry, 1978). Therefore, *Wnt* gene activity may be acting to expand a population of neuronal precursor cells or establish positional identity within the spinal cord. The expression patterns established in the spinal cord by 9.5 days generally persist at least through 14.5 days of embryogenesis (Wilkinson et al., 1987; Roelink and Nusse, 1991; data not shown). Even as various regions of the spinal cord begin to differentiate, *Wnt* gene expression is primarily confined to the mitotically active ventricular zone (data not shown). *Wnt*-4 expression in the spinal cord presents an intriguing and unique profile, as it encompasses distinct dorsal and ventral domains. Its appearance in the floor plate region is especially interesting (also see McGrew et al., 1992). Signals from the floor plate are believed to mediate axonal outgrowth, control cell differentiation, and participate in establishing dorsal-ventral polarity in the spinal cord (Tessier-Lavigne et al., 1988; Yamada et al., 1991).

There are striking dorsal-ventral restrictions of *Wnt* gene expression that are maintained throughout most of the CNS. These restrictions, most clearly observed in the spinal cord as three different patterns of *Wnt* gene expression, may distinguish different cell groups along the dorsal-ventral axis. *Wnt*-1, *Wnt*-3, and *Wnt*-3a are expressed along the dorsal midline, *Wnt*-4 in a broad dorsal domain (in addition to the floor plate), and *Wnt*-7a and *Wnt*-7b are expressed in the ventral half of the spinal cord. This variation along the dorsal-ventral axis at 9.5 days precedes the emergence of any differentiated cell types. Neural crest cell precursors are migrating from the dorsal midline at this time. Dorsal regions of the spinal cord will eventually give rise to sensory interneurons and ventral areas to motor neurons (reviewed by Altman and Bayer, 1984). Therefore, the dorsal-ventral variations in *Wnt* gene expression at 9.5 days may reflect important differences in neuronal precursor cell populations.

Limb development

Wnt gene expression is detectable in the limb bud from the time of the structure's emergence. A variety of studies indicate that polarities along the three limb axes (anterior-posterior, proximal-distal, and dorsal-ventral) are established independently of one another (reviewed by Tabin, 1991). Along the anterior-posterior axis, patterns of gene expression and experimental manipulations indicate that retinoic acid and the *Hox*-4 genes can influence axis determination (Dollé et al., 1989; Izpisua-Belmonte et al., 1991; Nohno et al., 1991; Morgan et al., 1992). However, we have found no correlation between the expression of known *Wnt* genes and anterior-posterior axis formation.

The proximal-distal gradient in *Wnt*-5a expression in the limb mesenchyme and ectoderm suggests that *Wnt* genes may contribute to patterning along this axis (also see Gavin et al., 1990). The possible involvement of homeobox genes in proximal-distal axis determination is indicated by the graded expression pattern of the *CHox*-1 gene cluster along this axis of the chicken limb (Yokouchi et al., 1991). Similarly, *Hox*-7.1 and *Hox*-8.1 are normally expressed in the distal limb mesoderm, and their transcriptional activation is an early event in the respecification of proximal-distal identity when portions of the proximal limb bud are grafted to a distal site (Davidson et al., 1991). Interestingly, the highest levels of *Wnt*-5a expression are localized to the progress zone, the distal domain of mitotic precursors that form the different limb structures.

Less is known about the mechanisms of dorsal-ventral axis specification, although the limb ectoderm appears to determine polarity along this axis (MacCabe et al., 1974). Therefore, the localized expression of *Wnt*-5a expression in the ventral half and *Wnt*-7a expression in the dorsal half of the ectoderm is especially interesting. It should be recalled that these two genes have different activities in mammary transformation assays and perhaps in other systems (see above). The overlap of *Wnt*-5a and *Engrailed*-1 (*En*-1) expression in the ventral ectoderm is intriguing in light of the possible interaction between *Wnt*-1 and *En*-1 in brain development (Davis et al., 1991; McMahon et al., 1992; see below). *Wnt*-7a and *Wnt*-5a clearly provide useful early markers for investigating the establishment of dorsal-ventral asymmetries in the limb primordia.

Molecular regulation of *Wnt* signalling

It will be interesting to determine how *Wnt* gene products cooperate with other growth factors and transcription factors to regulate vertebrate development. At the moment, little is known about the targets of Wnt protein action or the transcription factors regulating *Wnt* gene expression. It seems that the murine *Wnt*-1 and *En*-1 gene products may be part of a signalling pathway analogous to their *Drosophila* counterparts, *wingless* and *engrailed* (McMahon et al., 1992). Considerable attention has been given to the possible roles of the *Hox* genes in regulating development in the hindbrain, vertebrae, and limbs (Dollé et al., 1989; Wilkinson et al., 1989; Chisaka and Capecchi, 1991; Chisaka et al., 1992; Hunt et al., 1991; Izpisua-Belmonte et al., 1991; Kessel and Gruss, 1991; Lufkin et al., 1991). In the brain, most of the *Wnt* genes are expressed more anteriorly than the *Hox* genes. Thus other transcription factors, such as non-*Hox* cluster homeobox genes (Murtha et al., 1991; Price et al., 1991; Singh et al., 1991), Pou-domain genes (He et al., 1989), or the *Pax* gene family (Goulding et al., 1991; Jostes et al., 1991; Krauss et al., 1991; Walther and Gruss, 1991), may be regulating *Wnt* gene expression in these regions. The expression patterns of the homeobox genes related to the *Drosophila* genes *empty spiracles* and *orthodenticle* are especially intriguing (Simeone et al., 1992a,b). In early somite stage embryos, these genes are transcribed in overlapping domains in the forebrain and midbrain, much like the *Wnt* gene patterns described in this paper. Therefore, it is possible that these genes may regulate, or be regulated by, *Wnt* gene family members.

On the basis of the detailed descriptions of *Wnt* gene expression presented here, the functions of *Wnt* signalling are likely to be complex. However, these studies will prove essential in deciphering the roles that *Wnt* genes play in mouse development.

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