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 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-7a 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 Capecci, 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. Krulfluaf, 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 hybridizations 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 mammalian 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 35S-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 EcoRI-BamHI (5′ untranslated and coding); Wnt-3, 380 bp PCR product (coding); Wnt-3a, 775 bp EcoRI-BglIII (3′ untranslated); Wnt-4, 420 bp Smal-Smal (coding and 3′ untranslated); Wnt-5a, 360 bp PCR product (coding); Wnt-6, 336 bp EcoRI-XhoI (coding); Wnt-7a, 400 bp Smal-EcoRI (coding and 3′ untranslated); Wnt-7b, 306 bp PstI-EcoRI (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, 50 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 MgCl2, 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 SalI-BglII; 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.
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. 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.
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. 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).
**Wnt** in embryonic CNS and limb buds

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. 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.
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-
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 Carney, 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.
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 targeting 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-
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; Izipisu-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 mammalian 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; Izipisu-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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