Expression of two members of the *Wnt* family during mouse development—restricted temporal and spatial patterns in the developing neural tube

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The *Wnt* gene family encodes a group of cysteine-rich proteins implicated in intercellular signaling during several stages of vertebrate development. This family includes *Wnt-1* and *Wnt-3*, both discovered as activated oncogenes in mouse mammary tumors. Here we describe the molecular cloning of an additional member of the *Wnt* family, called *Wnt-3A*, and the spatial and temporal expression pattern of this gene as well as that of its close relative *Wnt-3*. The putative amino acid sequences of both proteins are almost 90% identical, but in situ hybridization to mouse embryo sections showed highly restricted patterns of expression of *Wnt-3* and *Wnt-3A*, largely in separate areas in the developing nervous system. In the spinal cord *Wnt-3* was expressed at low levels in the alar laminae and in the ventral horns, whereas *Wnt-3A* expression was confined to the roof plate. In the developing brain *Wnt-3* was expressed broadly across the dorsal portion of the neural tube with a rostral boundary of expression at the diencephalon. In contrast, *Wnt-3A* was expressed in a narrow region very close to the midline; expression extended into the bifurcating telencephalon, in a highly localized fashion. Both *Wnt-3* and *Wnt-3A* were expressed in the ectoderm, and *Wnt-3A* was also expressed in the periumbilical mesenchyme. Characteristic expression patterns of these two closely related genes suggest that *Wnt-3* and *Wnt-3A* play distinct roles in cell–cell signaling during morphogenesis of the developing neural tube.

[Key Words: *Wnt* family of genes; developmental expression; in situ hybridization]

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The classical description of the developing mouse embryo has provided a framework by which to study mammalian development at a molecular level. Among several different strategies for identifying genes that control mammalian embryogenesis, two approaches have proven particularly rewarding in recent years. Of the many genes identified by virtue of their ability to promote tumor formation when mutated or activated, several have been implicated in the control of the fundamental steps of cellular differentiation (Jakobovits et al. 1986; Wilkinson et al. 1989), leading to the suggestion that these proto-oncogenes have normal functions during development (Nusse 1988). Second, a steadily increasing number of mammalian genes are being identified because of their sequence similarity to genes known previously to control developmental decisions in *Drosophila* and/or *Caenorhabditis elegans*. In many cases, ontogenic function, as well as sequence similarity, appears to be evolutionarily conserved. Identification and isolation of the mouse *Wnt* gene family is a successful example of both approaches.

The *Wnt-1* (*int-1*) (Nusse and Varmus 1982) and *Wnt-3* (Roelink et al. 1990) genes were first recognized as proto-oncogenes due to their activation in mouse mammary tumors as a consequence of nearby insertion of mouse mammary tumor virus (MMTV) proviral DNA. It was subsequently shown that the *Drosophila* homolog of *Wnt-1* is *wingless*, a segment polarity gene implicated in a cascade of cell–cell communication events that establish developmental compartments in the early embryo (Rijsewijk et al. 1987). Additional members of the *Wnt* family are *Wnt-2* (*irp*), detected by its proximity to a CpG island in the vicinity of the human CFTR gene (Wainwright et al. 1988), and at least six other members identified by enzymatic amplification using degenerate primers (Gavin et al. 1990).

Biochemical studies of the mouse *Wnt-1* protein show that it is glycosylated and secreted (Papkoff et al. 1987) but remains tightly associated with the cell surface (Papkoff and Schryver 1990) and/or extracellular matrix (Bradley and Brown 1990). These observations are supported by genetic and biochemical analysis of the *wingless* gene

product, which acts in a cell nonautonomous fashion (Morata and Lawrence 1977) over relatively short distances of several cell diameters (Van den Heuvel et al. 1989). Sequence comparison among pairs of *Wnt* gene family members reveals ~45% similarity at the amino acid residue level, as well as complete conservation of 22 cysteine residues, a potential amino-terminal-linked glycosylation site, and the presence of a signal peptide. It therefore seems likely that all members of the *Wnt* gene family are involved in aspects of cell–cell communication.

Do different members of the *Wnt* gene family serve redundant or exclusive roles during mammalian embryogenesis? Functional redundancy may be a general hallmark of developmental gene families and is supported by the multiplicity and sometimes overlapping expression pattern of mouse *Hox* genes (Graham et al. 1989) and the minimal phenotypic consequences of inactivating the mouse *en-2* gene (A. Joyner, pers. comm.). Although the mouse *Wnt-1* gene is expressed along the entire spinal cord, its inactivation produces defects in development of the midbrain and the cerebellum only (McMahon and Bradley 1990; Thomas and Capecchi 1990). To address these issues and to better understand the role of *Wnt* genes in mouse development, we have characterized the expression patterns of *Wnt-3* and a new closely related gene, *Wnt-3A*. In contrast with *Wnt-1*, we find that *Wnt-3* and *Wnt-3A* are both expressed in the developing forebrain. Many areas are highly delimited and only partially overlap with each other, which suggests that *Wnt-1*, *Wnt-3*, and *Wnt-3A* have unique roles in morphogenesis of the developing neural tube.

Results

Cloning of Wnt-3A cDNA clones

During the isolation of *Wnt-3* cDNA clones from a day 8.5 mouse embryo cDNA library (the gift of B. Hogan) with a genomic probe (pBG87) containing the third and part of the fourth exon of the *Wnt-3* gene (Roelink et al. 1990), strongly and weakly hybridizing plaques were identified under high-stringency conditions. The strongly hybridizing plaques represented cDNA clones of *Wnt-3* itself. Five weakly hybridizing plaques were isolated and sequenced. All five cDNA inserts represented transcripts derived from the same gene, called *Wnt-3A*. One cDNA clone contained a complete putative open reading frame, which was colinear with the amino acid sequences of the other *Wnt* proteins (Fig. 1).

The putative *Wnt-3A* translation product is 91% identical to the *Wnt-3* protein sequence, disregarding the nonconserved amino-terminal signal peptide. The cysteine pattern in both proteins is identical. Many of the remaining differences between the *Wnt-3* and *Wnt-3A* protein sequences were substitutions of similar amino acid residues. The *Wnt-3A* protein had an amino-terminal signal sequence and two possible N linked glycosylation sites, one of which is conserved in all members of

the *Wnt* family. The conceptual primary translation product of *Wnt-3A* contains 352 amino acid residues, with a predicted molecular mass of 39,257 daltons. As the cDNA sequences in the 3'- and 5'-untranslated parts do not show any significant similarity, we used 3'-untranslated fragments as gene-specific probes specifically to detect expression of *Wnt-3* and *Wnt-3A*.

Expression of Wnt-3 and Wnt-3A during development

By RNA blot analysis we found a *Wnt-3A* transcript of 3.1 kb in adult lung but not in liver, heart, spleen, kidney, testis, skeletal muscle, mammary gland, brain, and uterus (data not shown). In embryos, the first detectable expression of *Wnt-3* and *Wnt-3A* was observed around day 10 of embryogenesis. As the *Wnt-3* and *Wnt-3A* cDNA clones were isolated from a day 8.5 mouse cDNA library, we expected low levels of expression earlier than day 10, but we have been unable to detect significant expression, either by RNA blot analysis or by in situ hybridization.

Serial transverse and sagittal sections of day 10.5–12.5 embryos were used to determine the exact areas of *Wnt-3* and *Wnt-3A* expression by in situ hybridization. We performed control sense strand hybridizations to sections adjacent to those presented and never observed any signal above background.

Wnt-3 and Wnt-3A expression in the day 10.5 embryo

The neural tube of a day 10.5 embryo consists of a number of morphologically distinct regions. From caudal to rostral, these regions are the spinal cord, the myelencephalon (hindbrain), the mesencephalon (midbrain), the diencephalon, and the two bilateral chambers of the telencephalon. Because of the strong curvature of the embryos, transverse sections can pass twice through the neural tube.

We found *Wnt-3* expression in the dorsal portion of all regions of the neural tube except in the telencephalon and in the hindbrain. In the hindbrain, *Wnt-3* was expressed in the junction between the neuroepithelium and the thin roof of ependymal cells (superior membrane) (Fig. 2A,C). More posterior and anterior, these regions merge; expression of *Wnt-3* encircled the complete superior membrane of the myelencephalon. *Wnt-3* was broadly expressed in the dorsal portion of the midbrain and diencephalon. The rostral boundary of *Wnt-3* expression lies in the telencephalic flexure of the prosencephalon, which marks the division between the developing diencephalon and telencephalon (Fig. 2A). The *Wnt-3* expression in the neural tube was continuous in length.

Like *Wnt-3*, the *Wnt-3A* gene was expressed in the dorsal portion of the neural tube but within a much smaller region. In the spinal cord, *Wnt-3A* was expressed only in the developing roof plate. Anteriorly, this localized midline expression bifurcates to accommodate the superior membrane of the rhombencephalon; in this area, expression of *Wnt-3* and *Wnt-3A* was coincident,

Developmental *Wnt-3* and *Wnt-3A* expression

100
Wnt-3A TACGGTCAAG GCAGAGGGCC CAGCGCCACT GCAGCCGCGC CACCTCCCAG GGCCGGGCCA GCCCAGGCGT CCGCGCTCTC GGGGTGGACT CCCCCCGCTG
200
CGCGCTCAAG CCGGCGATGG CTCCTCTCGG ATACCTCTTA GTGCTCTGCA GCCTGAAGCA GGCTCTGGGC AGCTACCCGA TCTGGTGGTC CTGGGCTGTG
Wnt-3A M A P L G Y L L V L C S L K Q A L G S Y P I W W S L A V
Wnt-3 M E P R L L G L L . G L . L S G T R V . A G L
300
GGACCCAGT ACTCTCTCT GAGCACTCAG CCCATTCTCT GTGCCAGCAT CCCAGGCGCTG GTACCGAAGC AGCTGCGCTT CTGCAGGAAC TACGTGGAGA
Wnt-3A G P Q Y S S L S T Q P I L C A S I P G L V P K Q L R F C R N Y V E I
Wnt-3 . Q . . T . . A S . . L . . G I . .
400
TCATGCCAG CGTGGCTGAG GGTGTCAAAG CGGGCATCCA GGAGTGCCAG CACCAGTTC GAGGCCGGCG TTGGAAGTGC ACCACCGTCA GCAACAGCCT
Wnt-3A M P S V A E G V K A G I Q E C Q H Q F R G R R W N C T T V S N S L
Wnt-3 L I D D . .
500
GGCCATCTTT GGCCCTGTTC TGGACAAAGC CACCCGGGAG TCAGCCTTTG TCCATGCCAT CGCCTCCGCT GGAGTAGCTT TCGCAGTGAC ACCTCTCTGT
Wnt-3A A I F G P V L D K A T R E S A F V H A I A S A G V A F A V T R S C
Wnt-3 .
600
GCAGAGGGAT CAGCTGCTAT CTGTGGGTGC AGCAGCCGCC TCCAGGGCTC CCCAGGCGAG GGCTGGAAGT GGGGCGGCTG TAGTGAGGAC ATTGAATTTG
Wnt-3A A E G S A A I C G C S S R L Q G S P G E G W K W G G C S E D I E F G
Wnt-3 . . . T S T D . H H K . P A D . .
700
GAGGAATGGT CTCTCGGAG TTTGCCGATG CCAGGAGAG CCGGCCGGAT GGCCGCTCTG CCATGAACCG TCACAACAAT GAGGCTGGGC GCCAGGCCAT
Wnt-3A G M V S R E F A D A R E N R P D A R S A M N R H N N E A G R Q A I
Wnt-3 V L . K T T . .
800
CGCCAGTCAC ATGCACCTCA AGTGCAAATG CCACGGGCTA TCTGGCAGCT GTGAAGTGAA GACCTGCTGG TGGTGCAGC CGGACTCCCG CACCATCGGG
Wnt-3A A S H M H L K C K C H G L S G S C E V K T C W W S Q P D F R T I G
Wnt-3 L D . A A . .
900
GATTTCCTCA AGGACAAGTA TGACAGTGCC TCGGAGATGG TGGTAGAGAA ACACCGAGAG TCTCGTGGCT GGGTGGAGAC CCTGAGGCCA CGTTACACGT
Wnt-3A D F L K D K Y D S A S E M V V E K H R E S R G W V E T L R P R Y T Y
Wnt-3 . A K . A L
1000
ACTTCAAGGT GCCGACAGAA CGCGACCTGG TCTACTACGA GGCTCACCC AACTTCTGCG AACCTAACCC GGAACCCGCG TCCTTCGGGA CGCGTGACCG
Wnt-3A F K V P T E R D L V Y Y E A S P N F C E P N P E T G S F G T R D R
Wnt-3 . . P N
1100
CACCTGCAAT GTGAGCTCGC ATGGCATAGA TGGGTGCGAC CTGTTGTGCT GCGGGCGCGG GCATAACGCG CGCACTGAGC GACGAGGGA GAATGACCA
Wnt-3A T C N V S S H G I D G C D L L C C G R G H N A R T E R R R E K C H
Wnt-3 T T . . . K . K
1200
TGTTGTTTCC ATTGGTGTCT CTACGTCAGC TGCCAGGAGT GCACACGTGT CTATGACGTG CACACCTGCA AGTAGGAGAG CTCCTAACAC GGGAGCAGGG
Wnt-3A C V F E W C C Y V S C Q E C T R V Y D V H T C K *
Wnt-3 I . I * *

Figure 1. Nucleotide sequence of the first 1200 bases of clone 15A representing *Wnt-3A* transcripts. The putative amino acid sequence of the *Wnt-3* protein is given in single-letter code. The asterisk (*) indicates the stop codon. Below the *Wnt-3A* amino acid sequence, the identical residues (dots) and nonidentical residues (single-letter code) of the *Wnt-3* amino acid sequence are shown.

although *Wnt-3A* expression was confined to a smaller area (Fig. 2B,D). Rostral to the myelencephalon, *Wnt-3A* was again expressed in the dorsal midline in the position of the developing cerebellum. Moving rostral still farther, midline expression of *Wnt-3A* again bifurcated at the telencephalic flexure and was expressed in small areas of the bilateral developing telencephalic bulbs. (Fig. 2D).

Outside the neural tube, *Wnt-3* was expressed in the dorsal ectoderm, the branchial arches, and the limb buds (Fig. 2A,C), and *Wnt-3A* was expressed in the mesenchyme tissue surrounding the umbilical veins (Fig. 2B).

Wnt-3 and *Wnt-3A* expression in the day 11.5 embryo

At day 11.5, the telencephalic bulbs have expanded and the telencephalic flexure has deepened. The developing

dorsal thalamus is still a relatively thin layer of cells, although some distinct expanding areas are evident. On midsagittal and parasagittal sections, the expression patterns of *Wnt-3* and *Wnt-3A* were generally similar to that seen at 10.5 days. The *Wnt-3* gene was broadly expressed in the dorsal portion of the neural tube with a rostral boundary in the dorsal thalamus. The anterior edge of the boundary was very sharp, just caudal of the telencephalic flexure (Fig. 3I), but the lateral edges were indistinct (Fig. 3G,J). In the hindbrain, expression continued to surround the roof of the myelencephalon (Fig. 3A,D,G,J,M).

Within the telencephalon, *Wnt-3A* was highly expressed in areas just dorsolateral of the telencephalic flexure (Fig. 3H,K,N). On midsagittal sections, *Wnt-3A* expression was also seen in a narrow band along the dorsal midline (Fig. 3B) that extended nearly to the rostral

Figure 2. Dark-field photomicrographs of day 10.5 mouse embryo sagittal (A and B) and transverse (C and D) sections, hybridized with *Wnt-3* (A and C) and *Wnt-3A* (B and D) antisense RNA probes. White areas and lines represent expression; grayness of the tissue is caused by aspecific light scattering. (Te) Telencephalon; (Di) diencephalon; (Me) mesencephalon; (My) myelencephalon; (SC) spinal cord; (Um) umbilicus; (MA) mandibular arch; (HL) hind limb.

tip of the neuraxis. Within the diencephalon, *Wnt-3A* was expressed in a small area at the boundary between the dorsal and ventral thalamus (Fig. 3K). *Wnt-3A* expression continued to surround the myelencephalic roof but appeared much closer to the midline than *Wnt-3* expression (Fig. 3B,E,H,K,N).

Wnt-3 and Wnt-3A expression in the day 12.5 embryo

At day 12.5, the same regions in the neural tube described for day 10.5 embryos can still be identified. The dorsal thalamus has grown significantly, and expression is easier to interpret because of the larger size of the embryos.

In the spinal cord, cells have differentiated further, and more functional areas can be distinguished. Transverse sections of the spinal cord showed a broad area of *Wnt-3* expression in the alar laminae of the spinal cord, similar to that seen in day 10.5 embryos. In addition, *Wnt-3* was expressed in the ventral horns, the area where the cell bodies of differentiating motor neurons are located (Fig. 5A). Expression in the ventral horns was also detected in a few consecutive sagittal sections (Fig. 5C). In the developing brain, *Wnt-3* was strongly expressed in the dorsal thalamus (Fig. 4A,C,E). Sagittal sections showed a rostral boundary of *Wnt-3* expression just behind the telencephalic flexure, in addition to a low level of expression in the dorsal portion of the mesencephalon and the cerebellum (Fig. 4A). In the hindbrain around the myelencephalic roof, *Wnt-3* expression has decreased sharply compared with that seen in younger embryos.

Expression of *Wnt-3A* in the spinal cord in day 12.5 embryos was confined to a triangle-shaped group of cells that form the presumptive roof plate (Fig. 5B). In the

brain, expression was observed in the dorsal midline up to the diencephalon (Fig. 4D), except in the hindbrain where, as at earlier times, a band of midline expression bifurcated to accommodate the diencephalic roof (Fig. 5D). In the diencephalon, the line of *Wnt-3A* expression bifurcated again (Fig. 4F) to extend into the medial walls of the telencephalon (Fig. 4D,F). Within the diencephalon, a small area of *Wnt-3A* expression was observed in both sagittal and transverse sections that marked the boundary between the dorsal and ventral thalamus (Fig. 4B,D), but expression was never extended into ventral structures. *Wnt-3A* expression proved highest around the dorsal portion of the telencephalic flexure (Fig. 4B). On transverse sections, it was clear that *Wnt-3A* expression surrounded the lateral and posterior choroid plexus (Figs. 4F and 5D).

At day 12.5, outside of the nervous system *Wnt-3* and *Wnt-3A* were expressed generally throughout the ectoderm. Relatively low levels were observed in the ectoderm covering the backs; higher levels were found in the ectoderm covering the limb buds and in the caudal position of the ectoderm covering the mandibular arch (Fig. 5E,F). The epithelium lining the forming oral cavity did not express *Wnt-3* and *Wnt-3A*. Within the mandibular arch, *Wnt-3* and *Wnt-3A* expression patterns were very similar, although higher for *Wnt-3*. In addition, *Wnt-3A* expression extended slightly further into the oral cavity.

Discussion

Expression of Wnt-3 and Wnt-3A in the neural tube

A number of gene families have been implicated in establishing compartments in the brain, based on their expression patterns: They are the *Hox* (Graham et al. 1989), *Pax* (Chowdhury et al. 1988), and *En* (Davis and Joyner 1988) gene families. Most of these genes, however, are expressed in the ventral portion of the neural tube and have their most anterior boundary of expression in the spinal cord or in the hindbrain (Kessel and Gruss 1990). This contrasts with *Wnt-3* and *Wnt-3A*, which are expressed in the dorsal portion of the neural tube and have their most anterior boundaries of expression in the diencephalon and the telencephalon, suggesting that no interaction of these genes and/or their products occurs.

Wnt-3A is expressed very strongly in the dorsal portion of the boundary between the diencephalon and both cerebral hemispheres. This area is part of the archicortex, a phylogenetically older system than the neocortex, and includes structures involved in emotional and affective behavior such as the hippocampus and the amygdala. Division of the prosencephalon and diencephalon is a relatively recent event in vertebrate evolution, and it seems possible that duplication of an ancestral gene giving rise to the *Wnt-3* and *Wnt-3A* genes occurred coincident with this division. If so, both genes may serve similar processes from both morphogenetic and phylogenetic perspectives. On the basis of our results, this process probably involves cell–cell signaling within the dorsal thalamus and dorsal archicortex.

Figure 3. Dark-field photomicrographs of day 11.5 mouse embryo sagittal sections, hybridized with *Wnt-3* (A, D, G, J, and M) and *Wnt-3A* (B, E, H, K, and N) antisense RNA probes. C, F, I, L, and O are bright-field photographs of the sections hybridized with *Wnt-3A*. From top to bottom, mid-sagittal to lateral sagittal sections are shown. (Te) Telencephalon; (DT) dorsal thalamus; (Me) mesencephalon; (FI) foramen inter-ventriculare; (My) myelencephalon. All photographs were taken at the same magnification.

In the spinal cord, *Wnt-3* is expressed in the ventral horns concomitant with motor neuron differentiation (Wentworth 1984). We speculate that the *Wnt-3* protein might serve as a chemotropic axon-guidance molecule. Alternatively, the *Wnt-3* protein might support growth and differentiation of neuron or glia cells. Despite the expression of the *Wnt-3* gene in the dorsal portion of the spinal cord, we have never observed any *Wnt-3* expression in cells or tissue derived from the neural crest.

We are struck by the expression of *Wnt-3A* along the dorsal midline of the entire neuraxis, sparing only the myelencephalic roof. Possibly, *Wnt-3A* may play a role in establishing the dorsal midline and/or serving as a reference point for further dorsoventral differentiation. In the chick spinal cord, both the roof plate and the floor plate elaborate signals necessary for the proper dorsoventral differentiation (Wagner et al. 1990). The presumed properties of the Wnt proteins as short-range cell-cell signaling molecules could well be the source of such differentiation signals and could easily act in the brain as well as the spinal cord. Furthermore, it seems possible that additional, as yet undiscovered, *Wnt* genes may be the source of floor plate signals.

These questions can be addressed most conveniently by manipulating the site of *Wnt-3A* expression, either via transgenic animals or by transplantation experiments coupled with *in situ* hybridization. Both approaches are feasible, although the transplantation studies are best addressed in the chick.

Expression of Wnt-3 and Wnt-3A outside the neural tube

Within the mesodermally derived tissues, only *Wnt-3A* is expressed in the mesenchyme surrounding the umbilical veins in the day 10.5 embryo. This area is coincident with that described for *Wnt-2* expression (McMahon and McMahon 1989). Within the ectoderm, *Wnt-3* and *Wnt-3A* are expressed most highly in the caudal portion of the mandibular arches. Ferguson (1988) has speculated that the ectoderm plays an important role in establishing the caudal/rostral identity of the branchial arches by generating inductive signals. The products of *Wnt-3* and *Wnt-3A* might help to establish the differences between the rostral (bone and teeth forming) and caudal side of the mandibular arch. The onset of *Wnt-3* expression in the

Figure 4. Dark-field photomicrographs of transverse (*C*, *D*, *E*, and *F*) and sagittal (*A* and *B*) sections of the brain of day 12.5 mouse embryo hybridized with *Wnt-3* (*A*, *C*, and *E*) and *Wnt-3A* (*B*, *D*, and *F*) antisense RNA probes. White areas represent expression, grayness of the tissue is caused by light scattering in the tissues. (Te) Telencephalon; (DT) dorsal thalamus; (Di) diencephalon; (Me) mesencephalon; (Ce) cerebellum; (LC) lateral choroid plexus. All photographs were taken at the same magnification.

branchial arch ectoderm is much earlier and stays at much higher levels than *Wnt-3A* expression.

The Wnt family of genes

The *Wnt-1* gene now appears to be part of a large gene family that consists of at least 10 members in the mouse. Apart from the novel member, *Wnt-3A*, reported here, some six other related genes have been identified recently through polymerase chain reactions (PCRs) with degenerate primers (Gavin et al. 1990). From the expression pattern of some of these genes it has been inferred that they play important roles during differentiation of several organs in midgestation embryos, particularly in the developing nervous system. For *Wnt-1*, the prototype member of this family, such an essential function has recently been demonstrated by generating mouse strains that lack a functional *Wnt-1* allele. These homozygous insertional null mutations of the *Wnt-1* gene affect the formation of those areas in the midbrain where a dorsoventral ring of *Wnt-1*-expressing cells is detected as well as adjacent cerebellar structures (McMahon and Bradley 1990; Thomas and Capecchi 1990). Compared with the pattern described for *Wnt-1* expression, *Wnt-3A* expression completely overlaps that of *Wnt-1* in several notable areas (Wilkinson et al. 1987a). In particular, *Wnt-*

1 expression in the roof plate and surrounding the myelencephalic roof is identical to *Wnt-3A* expression. If *Wnt-1* and *Wnt-3A* are functionally redundant proteins, this may explain why mice with an inactivated *Wnt-1* gene apparently have a normal spinal cord and hindbrain.

The expression patterns shown here suggest that the *Wnt-3* and *Wnt-3A* genes, like *Wnt-1*, might also play a role in the brain morphogenesis but at other areas. In particular, the rostral boundaries of *Wnt-3* and *Wnt-3A* expression are anterior to *Wnt-1* expression, which suggests that *Wnt-3* and *Wnt-3A* may control morphogenesis of areas that are most highly developed in humans.

Materials and methods

Tissue preparation

Timed pregnant BALB/c and CD-1 mice were identified by the appearance of the vaginal plug. Because matings took place around midnight, the age of the embryos was considered to be 0.5 day on the day of appearance of the vaginal plug. Embryos were removed at the desired ages and fixed overnight in 4% paraformaldehyde dissolved in PBS. The next day, the embryos were dried through an ethanol series and stored in 80% ethanol. Ethanol-dried, xylene-treated embryos were embedded in wax,

Figure 5. Dark-field microphotographs of transverse and sagittal sections of day 12.5 mouse embryo, hybridized with *Wnt-3* (*A*, *C*, and *E*) and *Wnt-3A* (*B*, *D*, and *F*) antisense RNA probes. (*A* and *B*) Transverse sections through the spinal cord; (*C*) a parasagittal section through the anterior part of the spinal cord; (*D*) a parasagittal section through the hindbrain and the cerebellum. (*E* and *F*) Parasagittal sections through the ventral part of the embryo. (Me) Mesencephalon; (RP) roof plate; (PC) posterior choroid plexus; (SC) spinal cord; (VH) ventral horn; (My) myelencephalon; (MA) mandibular arch; (T) tail; (HL) hind limb bud.

and ribbons of 6- μ m sections were cut on a microtome. Sections were transferred to 3-aminopropyltriethoxysilane-coated (Sigma) glass slides and stored desiccated at 4°C.

Organs for RNA preparation were dissected out, immediately frozen in liquid nitrogen, and stored at -70°C.

Isolation and subcloning of the *Wnt-3A* cDNA clones

Wnt-3A cDNAs were identified by hybridization of 5×10^6 recombinant phage of a day 8.5 mouse embryo cDNA library with probe pBG87 (Roelink et al. 1990) under high-stringency conditions (65°C, $3 \times$ SSC). Subsequent washes were performed in $0.1 \times$ SSC at 65°C. pBG87 contains the third and part of the fourth exon of the *Wnt-3* gene. Phage DNA was isolated according to established procedures (Sambrook et al. 1989), and the cDNA inserts were released from the phage arms with *Eco*RI. The isolated cDNA fragments were subcloned in the *Eco*RI site of pGEM-3Zf(+).

Wnt-3A cDNA sequencing

Wnt-3A clones 9A and 15A, the longest fragments obtained, were sequenced by using primers specific for the phage T7 and SP6 promoters as internal primers. All sequence reactions were performed on denatured double-stranded templates by using Sequenase version 2 according to the manufacturer's instructions. Both strands of clones 9A and 15A were sequenced. Other clones were identified on partial sequences. No other clones extended 5' or 3' over clone 9A or 15A. The *Wnt-3A* cDNA sequence including all available sequence 3' of the coding domain will appear in the EMBL and DDBJ Nucleotide Sequence Databases under accession number X56842.

In situ hybridization

In situ hybridizations were performed according to an established protocol (Wilkinson et al. 1987b). *Wnt-3* and *Wnt-3A* probes were derived from noncoding and nonconserved cDNA fragments. The *Wnt-3* probe is a 1.2-kb *Sall*-*Eco*RI fragment cloned in pGEM-3Zf(+). The *Wnt-3A* probe is a 750-bp *Eco*RI-*Bgl*II fragment also cloned in pGEM-3Zf(+). [³⁵S]UTP-labeled transcripts of the *Wnt-3* and *Wnt-3A* clones were generated using established procedures (Melton et al. 1984). Antisense *Wnt-3* transcripts were generated using T7 RNA polymerase on *Sall* linearized template; antisense transcripts of the *Wnt-3A* clone were generated using SP6 polymerase on an *Eco*RI linearized template. Probes were degraded to an average length of 100 bp by controlled alkaline lysis (Cox et al. 1984). Sections were hybridized at 59°C for 6–16 hr and washed according to Wilkinson et al. (1987b) at 60°C. Slides were dipped in Kodak NBT-2 emulsion, dried, and exposed at 4°C for 4–10 days. Exposure times were judged on overnight exposure to XAR2 film (Kodak). Slides were developed in D19 developer (Kodak), and the emulsion was fixed in 30% sodium thiosulfate. Sections were stained with methyl green, mounted, and examined and photographed using Kodak Tech Pan film, under dark-field illumination.

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Roelink and Nusse

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Expression of two members of the Wnt family during mouse development--restricted temporal and spatial patterns in the developing neural tube.

H Roelink and R Nusse

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