Expression of multiple novel *Wnt*-1/*int*-1-related genes during fetal and adult mouse development

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The mammary tumor-associated proto-oncogene *Wnt*-1/*int*-1 encodes a secreted protein implicated in the regulation of neural development in vertebrates and segmental pattern in *Drosophila*. Using a PCR-based strategy, we isolated cDNAs encoding six novel, related proteins that are expressed in fetal mice. Predicted proteins are of similar molecular masses (38–42 kD) and share between 50% and 85% of amino acids. All contain a putative hydrophobic signal sequence, and comparative analysis reveals 83 absolutely conserved amino acid residues, including 21 cysteines. Transcripts were detected throughout fetal development by Northern blot analysis. Detailed examination of the expression of two of these genes by in situ hybridization revealed complex temporal and spatial patterns of transcription. All new *Wnt* family members are expressed in adult tissues, particularly in brain and lung. These data support the view that the *Wnt*-1/*int*-1 family constitutes a large family of signaling peptides with diverse roles in mouse development.

[*Key Words*: *Wnt*-1/*int*-1-related genes; fetal expression; adult expression]

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For orderly development of the mammalian embryo, signaling between cells plays an important part in the regulation of patterning, growth, morphogenesis, and differentiation. It is tempting to speculate, by analogy with other vertebrate organisms, that peptide growth factor-like molecules will play an important part in mediating such interactions (Smith 1989; Ruiz i Altaba and Melton 1990). Many growth factors and their receptors are developmentally regulated and spatially localized within the conceptus (for review, see Mercola and Stiles 1988). However, the exact developmental roles of these molecules have yet to be determined. The diverse nature of the many different interactions that are occurring presumably requires a diverse array of signaling molecules. We have focused our attention on one of these, the *int*-1 gene, which is likely to play an important role in regulation of the development of vertebrate and invertebrate species. In line with others (Roelink et al. 1990), we have adopted a new nomenclature in which *int*-1 is now referred to as *Wnt*-1. Other family members also receive the designation *Wnt* to indicate their relationship to *Wnt*-1.

Wnt-1 was originally isolated from mouse mammary tumor virus (MMTV)-induced mammary tumors that arose following insertional activation of the *Wnt*-1 gene (Nüsse and Varmus 1982; Nüsse et al. 1984; van Ooyen and Nusse 1984). Analysis of the predicted open reading frame indicated that the 41-kD *Wnt*-1 protein contained a hydrophobic leader sequence and was probably secreted (van Ooyen and Nüsse 1984; Fung et al. 1985). A variety of studies have now demonstrated clearly that *Wnt*-1 protein enters the secretory pathway (Brown et al. 1987; Papkoff et al. 1987; McMahon and Moon 1989a), is processed and glycosylated (Brown et al. 1987; Papkoff et al. 1987; Papkoff 1989; Bradley and Brown 1990; Papkoff and Schryver 1990), and finally secreted (Papkoff 1989; Bradley and Brown 1990; Papkoff and Schryver 1990). However, it is likely that the protein acts only locally because in cell culture systems it is tenaciously bound to the extracellular matrix and/or cell surface and is rarely detected in the medium (Bradley and Brown 1990; Papkoff and Schryver 1990).

In the mouse (Shackleford and Varmus 1987; Wilkinson et al. 1987a), frog (Noordemeer et al. 1989), and zebra fish (A. Molven, pers. comm.), *Wnt*-1 is normally expressed during early neural development. In the mouse, expression is first localized to the presumptive midbrain and then becomes restricted to a subset of cells at the dorsal midline of the neural tube and in a circular band just rostral to the midbrain–hindbrain junction (Wilkinson et al. 1987a). However, expression is not restricted to vertebrates. The *Drosophila* homolog of *Wnt*-1 has been identified as the segment polarity gene *wingless* (Rijsewijk et al. 1987), a gene required for normal cuticular patterning in each segment (Nüsslein-Volhard and Wieschaus 1980). In *Drosophila*, *wingless* is secreted (van den Heuvel et al. 1989) and acts on neighboring cells to regulate at least one gene, the homeo-

box-containing segmentation gene *engrailed* (DiNardo et al. 1988). On the basis of the pattern-regulating role of *wingless* in *Drosophila*, we have suggested that vertebrate *Wnt*-1 may also play a role in patterning (McMahon and Moon 1989a). Recent mutational studies in which mice were generated carrying *null* alleles of *Wnt*-1 support this view (McMahon and Bradley 1990; Thomas and Capecchi 1990). In the absence of *Wnt*-1, specific brain regions are absent as early as 9.5 days of development (McMahon and Bradley 1990), indicating that *Wnt*-1 expression is essential for the normal organization of structures along the anterior–posterior axis of the developing central nervous system (CNS).

Until recently, the search for vertebrate *Wnt*-1-related genes that might regulate distinct developmental processes has been unsuccessful (A.P. McMahon, unpubl.). However, a human *Wnt*-1-related gene, *Wnt*-2/*irp* was identified by chance in a chromosomal walk directed around the cystic fibrosis locus (Wainwright et al. 1988). Our studies on the murine counterpart suggest that this gene also functions during early fetal development (McMahon and McMahon 1989). Moreover, the fact that there is no overlap in the expression of *Wnt*-1 and *Wnt*-2 strongly suggests that they are indeed regulating different events. A third *Wnt* gene, *Wnt*-3, which was isolated from several MMTV-induced mammary tumors (Roelink et al. 1990), also shows fetal expression, although the spatial organization has not been determined at present.

On the basis of a comparative analysis of *Wnt*-1 and *Wnt*-2 sequences, we have used a strategy based on the polymerase chain reaction (PCR) (Saiki et al. 1988) to clone six new members of the *Wnt* family from 8.5- to 9.5-day fetal mouse cDNA. The predicted proteins share many features in common with the *Wnt*-1 protein, including a hydrophobic amino terminus that presumably functions as a signal peptide sequence. Thus, the *Wnt*-1 family of putative signaling molecules consists of at least nine members, all of which are expressed in the developing mouse fetus.

Results

Cloning of Wnt-*1-related cDNAs*

Comparison of the published amino acid sequences for mouse (van Ooyen and Nüsse 1984; Fung et al. 1985), human (van Ooyen et al. 1985), *Drosophila* (Rijsewijk et al. 1987), and *Xenopus* (Noordemeer et al. 1989) *Wnt*-1, and the human (Wainwright et al. 1988) and mouse (McMahon and McMahon 1989) forms of the *Wnt*-1-related protein *Wnt*-2, reveals a number of short regions of complete amino acid conservation. The underlying conservation of nucleic acid sequences encoding these regions allows the design of degenerate oligonucleotide primers for PCR amplification of sequences containing these motifs (Libert et al. 1989). Using this approach, Kamb et al. (1989) have identified a short stretch of *Caenorhabditis elegans* genomic DNA related to *Wnt*-1. We have modified this strategy to search for new murine members of the *Wnt*-1 family. Degenerate oligonucleotide primers to several conserved regions were tested for their ability to amplify *Wnt*-1- and *Wnt*-2-containing plasmids. On the basis of these results (data not shown), two sets of degenerate primers were chosen (Fig. 1A). The 5′ set encompassed the absolutely conserved amino acid sequence QECKCH (Figs. 1A and 2, amino acids 258–263). The 3′ set encompassed the sequence FHWCC (Figs. 1A and 2, amino acids 391–395). *Xenopus* *Wnt*-1 shows an H to N amino acid substitution within this conserved region; however, the oligonucleotides used encoded only the more highly conserved histidine residue.

Amplification of plasmids containing mouse *Wnt*-1 and *Wnt*-2 cDNAs with the degenerate oligonucleotides generated the expected products of 434 and 406 bp, respectively (Fig. 1B, lanes 3 and 6). Digestion of the *Wnt*-1-amplified product with *Acc*I and the *Wnt*-2-amplified product with *Eco*RI reduced the amplification products to two bands of 233 and 201 bp (*Wnt*-1; Fig. 1B, lane 1), and 216 and 190 bp (*Wnt*-2; Fig. 1B, lane 5), as expected from the restriction maps of these cDNAs.

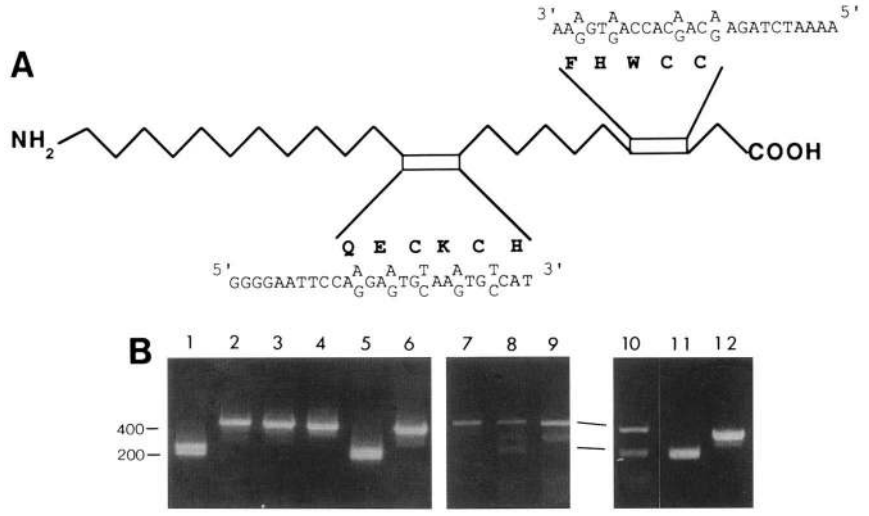

Figure 1. Amplification of 9.5-day fetal cDNA with degenerate oligonucleotide primers. (*A*) Conserved regions within the *Wnt*-1 sequence and degenerate PCR primers encoding these regions. (*B*) Restriction enzyme analyses of reaction products. Templates were as follows: (Lanes *1–3*) *Wnt*-1-containing plasmid; (lanes *4–6*) *Wnt*-2-containing plasmid; (lanes *7–10*) 9.5-day fetal cDNA; (lanes *11* and *12*) *Wnt*-1 and *Wnt*-2 plasmid mix. Amplified DNAs were digested with the following restriction enzymes: *Acc*I (lanes *1*, *4*, and *7*); *Eco*RI (lanes *2*, *5*, and *8*; *Eco*RI and *Acc*I (lanes *10* and *11*); undigested (lanes *3*, *6*, *9*, and *12*).

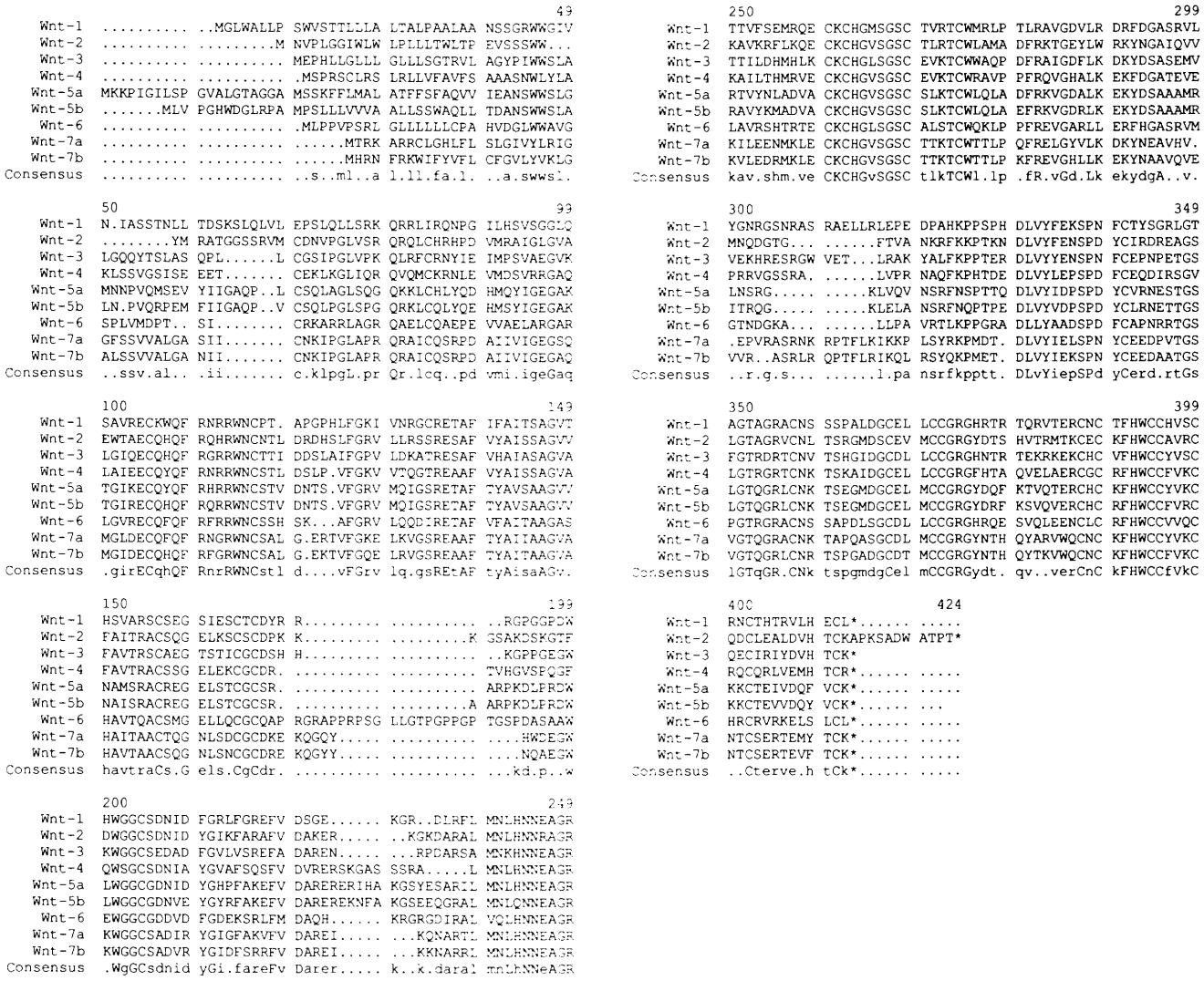

Figure 2. Alignment of the predicted protein sequences for the mouse *Wnt* family members. Full-length amino acid sequences encoded by the six novel *Wnt*-1-related cDNAs are aligned with those of mouse *Wnt*-1 and *Wnt*-2. Alignments were generated using the BESTFIT program (GCG) with default parameters. Composite alignments and consensus were generated using the LINE UP program (GCG), and final optimal alignments were obtained by manual editing.

To search for novel *Wnt*-1-related genes with a developmental role, these primers were used to amplify 9.5-day mouse fetal cDNA. At this time, both *Wnt*-1 and *Wnt*-2 are expressed, and many interesting processes are under way including gastrulation, somitogenesis, neurulation, and early limb and facial development. Amplification of the cDNA template generated major bands similar in size to *Wnt*-1 and *Wnt*-2 and a variable minor artifactual band. Under conditions that result in complete digestion of a control reaction (Fig. 1B, lane 11), a fraction of the amplified cDNA always remained undigested by *Acc*I and *Eco*RI (Fig. 1B, lane 10).

To determine whether this resistant fraction was due to specific amplification of *Wnt*-related sequences, reaction products were subcloned following digestion with *Eco*RI and *Xba*I, which cut in the 5′ and 3′ oligonucleotides, respectively. Eighty colonies were screened, and 28 were found to contain inserts >100 bp. Sequencing identified subclones containing *Wnt*-1 and *Wnt*-2, as expected (Table 1). In addition, four new *Wnt* clones were identified (Table 1) and designated *Wnt*-4, *Wnt*-5a, *Wnt*-6, and *Wnt*-7a. All four novel clones contained a single, long open reading frame with absolute conservation of all cysteine residues, a striking feature of the *Wnt* family (see Fig. 2).

To obtain cDNA clones encompassing the entire coding sequence of these genes and to verify the sequence predicted by the PCR products, an 8.5-day mouse fetal cDNA library was screened repeatedly using the cloned amplification products. Additional sequence for *Wnt*-6 and *Wnt*-7a was also obtained by isolating clones from a mouse neonatal brain library. In the process of screening the 8.5-day cDNA library, two new clones were identified, which cross-hybridized with either the

Table 1. *Insert distribution of* Wnt-*1-related PCR clones*

	No. of isolates[a]
Wnt-1/*int*-1	1
Wnt-2/*irp*	13
Wnt-4	1
Wnt-5a	2
Wnt-6	5
Wnt-7a	3

[a]Of 80 colonies screened, 28 contained inserts, 25 of which were *Wnt*-1-related.

Wnt-7a or *Wnt*-5a probes. These new clones, highly related to *Wnt*-7a and *Wnt*-5a, were designated *Wnt*-7b and *Wnt*-5b, respectively. Therefore, the combined approach of PCR amplification and library screening identified six novel *Wnt*-1-related genes.

Predicted amino acid sequence of Wnt *proteins*

The amino acid sequences predicted from the new *Wnt*-1-related cDNAs are aligned with the published sequences of *Wnt*-1 and *Wnt*-2 (Fig. 2). As with *Wnt*-1 and *Wnt*-2, all of the related proteins have a hydrophobic amino terminus, which may function as a signal peptide sequence. The unprocessed proteins range in length from 348 to 379 amino acids (Table 2), reflecting variability in the sequences, particularly at the poorly conserved amino terminus and at several internal sites (Fig. 2). Predicted molecular masses range from 38,944 to 42,105 daltons (Table 2).

There are no large blocks with particularly high conservation that might point to functionally conserved domains of the proteins; rather, with the exception of the amino terminus, short stretches of one to a few absolutely conserved amino acids are scattered throughout the protein. That the cysteine residues play a critical role in this family is attested to by the extreme conservation of these amino acids. All 21 cysteines present from amino acid 105 in the compilation (Fig. 2) are invariant. Initial sequence of a *Wnt*-7b cDNA clone from an 8.5-day embryo predicted an arginine and not a cysteine at the conserved cysteine position 166 (Fig. 2). However, as *Wnt*-7b genomic DNA encoded a cysteine (data not shown), the original cDNA clone presumably contained an error. Another 62 amino acids, particularly most of the glycine and tryptophan residues, are also absolutely conserved among all eight proteins.

Table 2. *Predicted length and molecular mass of* Wnt-*1-related proteins*

	Length (amino acids)	Molecular mass (daltons)
Wnt-4	351	39,005
Wnt-5a	379	42,105
Wnt-5b	372	41,549
Wnt-6	364	39,511
Wnt-7a	348	38,944
Wnt-7b	348	39,257

Interestingly, although the amino acid sequence encompassed by the 3′ degenerate oligonucleotide set was absolutely conserved (FHWCC; Fig. 2, 391–395), this was not the case for the 5′ set. All six new genes showed one or two divergent amino acids in the first two codon positions. It is clear that despite this added degeneracy in the nucleotide sequence and, therefore, mismatch of the primers, homology at the stretch encoding the four absolutely conserved amino acids (CKCH; Fig. 2, 260–263) was sufficient for efficient and specific PCR amplification.

Analysis of the homology matrix (Fig. 3) indicates several relationships. The pairs *Wnt*-7a and *Wnt*-7b and *Wnt*-5a and *Wnt*-5b are closely related. Within each pair, 87% of amino acids are identical, in contrast to the general level of identity that ranges between 49% and 61%. Interestingly, all proteins except *Wnt*-6 and *Wnt*-3 share greater homology with *Wnt*-2 than with *Wnt*-1. Furthermore, *Wnt*-2 and all of the other related proteins, but not *Wnt*-1 itself, show additional conservation of two cysteine residues toward the amino termini (Fig. 2, positions 70 and 84). In contrast, a cysteine residue present in mouse (Fig. 2, position 134; van Ooyen and Nüsse 1984; Fung et al. 1985), human (van Ooyen et al. 1985), *Xenopus* (Noordemeer et al. 1989), and *Drosophila* (Rijsewijk et al. 1987) *Wnt*-1 is not conserved in any of the related proteins. Therefore, thus far, *Wnt*-1 is the most divergent member of this family. Homology between *Wnt*-6 and other family members is also low, due in part to ~20 amino acids of additional sequence in the middle of the protein (Fig. 2). However, the additional conserved cysteines at the amino terminus suggest a closer relationship between *Wnt*-6 and other family members than to *Wnt*-1.

Fetal expression of the Wnt *genes*

To determine the developmental profiles of expression of the *Wnt*-1-related genes, RNA blot hybridization was performed with fetal RNAs. All probes were tested by Southern blot hybridization for specificity. No cross-hybridization, with even closely related genes, was seen with any of the probes. All six of the novel *Wnt* genes are expressed throughout fetal development from 9.5 to 17.5 days with maximum expression during the first half of fetal development (Fig. 4). *Wnt*-7a, *Wnt*-7b, and *Wnt*-5b give rise to single RNA species of ~4.4, 4.4, and 3.2 kb, respectively. In contrast, *Wnt*-4 and *Wnt*-6 each give rise to two transcripts of 4.7 and 1.6 kb, and 2.2 and 1.8 kb, respectively. *Wnt*-5a generates at least five distinct RNA species, ranging in size from 4.6 to >8 kb.

Analysis of multiple overlapping fetal cDNA clones suggests that much of the heterogeneity results from the use of alternative polyadenylation sites and/or alternative splicing or promoter usage that does not alter the predicted coding region. For example, four different polyadenylation sites are utilized by *Wnt*-6 transcripts (data not shown). Three are clustered closely together, and the fourth lies ~400 bp 3′, thereby generating the distinct 1.8- and 2.2-kb bands seen on Northern analysis.

	Wnt-1	*Wnt*-2	*Wnt*-3	*Wnt*-4	*Wnt*-5a	*Wnt*-5b	*Wnt*-6	*Wnt*-7a	*Wnt*-7b
Wnt-1		54%	56%	56%	52%	50%	51%	51%	52%
Wnt-2			56%	60%	58%	60%	51%	55%	57%
Wnt-3				60%	57%	58%	49%	55%	56%
Wnt-4					58%	57%	54%	59%	61%
Wnt-5a						87%	49%	56%	58%
Wnt-5b							50%	57%	58%
Wnt-6								53%	51%
Wnt-7a									87%
Wnt-7b									

Figure 3. Homology matrix indicating amino acid identities among *Wnt* family members. Sequences were analyzed using the DISTANCE program (GCG) excluding gaps.

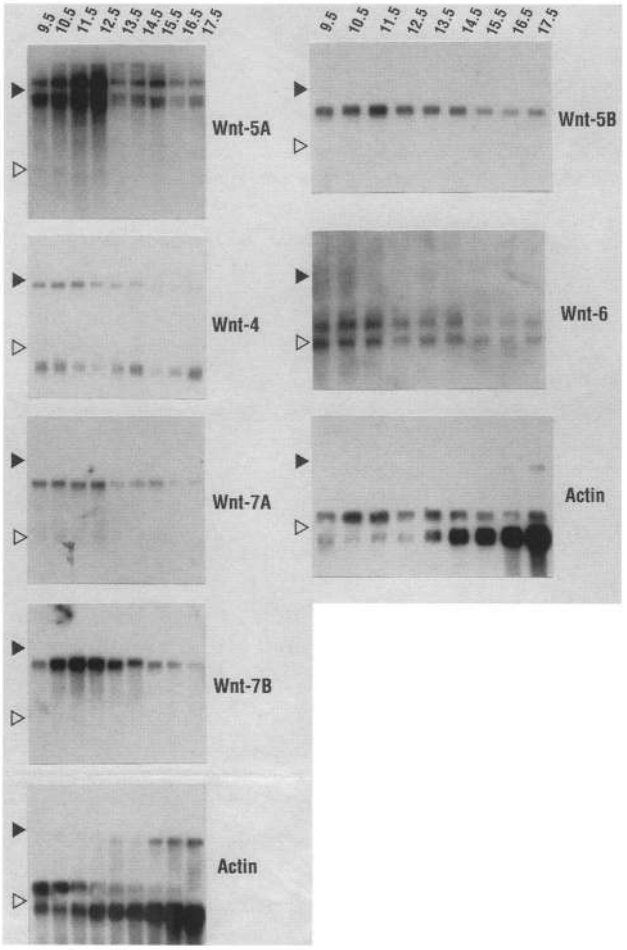

Figure 4. Expression of novel *Wnt*-1-related genes during fetal mouse development. Fetal RNAs from 9.5 to 17.5 days of development were analyzed by Northern blot hybridization. In addition, filters were probed with a β-actin probe (Minty et al. 1981) to verify similar loadings and integrity of RNA. As this probe cross-reacts with skeletal actins, a lower hybridizing band is seen to accumulate with time. (*Upper* band) The β-actin transcript. Some variability in the absolute amounts of poly(A) RNA loaded is apparent from early to late stages; thus, the variation in the levels of *Wnt* transcripts at different stages does not necessarily reflect absolute changes in RNA abundance with time. Solid and open arrowheads indicate 28S and 18S rRNAs, respectively.

Heterogeneity in *Wnt*-5a and *Wnt*-4 transcripts results from transcript variability 5′ to the initiation methionine. However, from the analysis of cDNA and genomic clones encoding *Wnt*-4, differential splicing is predicted to generate alternative *Wnt*-4 transcripts encoding different proteins. cDNA clones were isolated that encoded full-length *Wnt*-4 protein and a truncated form in which a 42-bp in-frame deletion removed 14 amino acids. The deleted sequence, GT**REAAFVYAISSA** (Fig. 2, amino acids 133–146), contains five amino acids (bold) that were absolutely conserved among all eight members of the *Wnt* family. Thus, it is possible that proteins with different functional roles are generated, presumably as a result of alternative splicing.

To examine *Wnt*-4 transcript diversity, genomic clones encompassing this region were isolated and sequenced. The genomic sequence indicates two splice acceptor sites separated by 42 nucleotides (Fig. 5). Transcripts using splice acceptor[1] encode full-length protein, while transcripts utilizing splice acceptor[2] delete the 42-nucleotide stretch encoding the 14 amino acid region (Fig. 5). As the polypyrimidine tract preceding the splice acceptor[2] is relatively poor, splicing to acceptor[1] and, therefore, production of RNA with full coding capability, might be expected to predominate. It will be interesting to examine the mechanism and consequences of these splicing events more closely.

Analysis of Wnt-*5 expression by in situ hybridization*

The expression of *Wnt* transcripts throughout fetal development suggests that *Wnt* genes may function at this time. To determine more precisely the expression profiles for two of these genes, *Wnt*-5a and *Wnt*-5b, we performed an extensive series of in situ hybridizations from 6.5 to 14.5 days. Although *Wnt*-5b transcripts were present at low levels throughout the embryo and fetus (data not shown), *Wnt*-5a transcripts showed complex spatial and temporal patterns of expression. For brevity, only data using antisense RNA probes are shown. Control experiments using sense RNA probes revealed no specific sites of artifactual signal.

At early gastrula stages, 6.5 days postcoitum, *Wnt*-5a transcripts were detected in the maternally derived decidual tissue that forms a capsule around the conceptus. Expression within the deciduum was localized exclusively to the mesometrial component (Fig. 6a,b), the area through which maternal blood vessels gain access to the

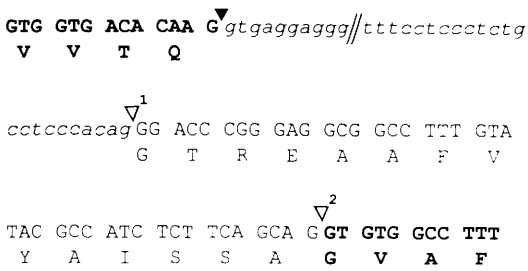

Figure 5. Genomic sequence surrounding alternatively spliced region of *Wnt*-4. (▽) Two splice acceptor sites; (▼) single splice donor.

uterus. In the embryo at this time, *Wnt*-5a expression was found in the trophectoderm giant cells and in the posterior ectoderm and mesoderm of the early gastrulating embryo (data not shown). High levels of *Wnt*-5a transcripts were detected in the posterior region, as late as 9.5 days (Fig. 6c,d). As in earlier stages, *Wnt*-5a transcripts show no specific tissue localization in the posterior region but were widely distributed in the neural ectoderm, the mesoderm, and the gut endoderm. Thus, in this region, *Wnt*-5a expression correlates with posterior position rather than with cell lineage. By 9.5 days, several other discrete sites of *Wnt*-5a expression were visible. In the developing CNS, *Wnt*-5a transcripts were present in the ventral portion of the entire midbrain (Fig. 6e,f). Expression was highest in this region of the CNS but was also detected elsewhere. In the forebrain and rostral hindbrain (metencephalon), no expression was detected; however, in the caudal hindbrain (myelencephalon, data not shown) and rostral spinal cord (Fig. 6i–l), *Wnt*-5a transcripts showed a ventral localization (excluding the floor plate) similar to that seen in the midbrain. Expression of *Wnt*-5a was seen in several populations of cranially derived neural crest cells, but within these populations *Wnt*-5a transcripts were restricted to a spatially defined subpopulation of cells. For example, frontal–nasal processes had high levels of *Wnt*-5a transcripts localized toward the tips of the facial processes (Fig. 6g,h) in the neural-crest-derived mesenchyme. At this stage, expression was also seen within the adjacent ectoderm. *Wnt*-5a expression remained associated with the frontal region of the face up until at least 14.5 days (data not shown, but see Fig. 8, below); but by this time, expression in the overlying ectoderm had ceased. In addition, the lateral but not the medial neural crest mesenchyme of the branchial bars expressed *Wnt*-5a at 9.5 (Fig. 6i,j) and 10.5 days (data not shown). Extensive expression of *Wnt*-5a was also seen in different populations of lateral mesoderm (Fig. 6k,l) along the body axis.

One of the most striking sites of *Wnt*-5a expression was in the developing limb. *Wnt*-5a transcripts were localized in the limb from early limb bud stages at 9.5 days to the late limb at 14.5 days. Expression was seen in both fore- and hindlimbs with a similar spatial organization. Expression in the developing forelimb is shown in Figure 7. At 9.5 days, expression was restricted to the slightly thicker ventral ectoderm of the limb bud (Fig. 7a,b). By 10.0 days, the proximal–distal axis of the limb bud had elongated considerably. At this time, *Wnt*-5a transcripts showed a graded distribution along the distal–proximal axis. Highest levels of *Wnt*-5a were found in the apical ectoderm at the distal tip of the limb, lower levels in the underlying distal mesenchyme, and very low levels in the proximal mesenchyme (Fig. 7c,d). By 11.5 days, expression was no longer seen in the apical ectoderm but remained associated with the distal mesenchyme from 11.5 to 14.5 days (Fig. 7e–j). In addition, at these later stages, *Wnt*-5a expression was also detected in association with condensing cartilage, but the bulk of *Wnt*–5a expression localized remained within the distal mesenchyme of the limb.

In situ analysis indicated that of the *Wnt*-5 genes, only *Wnt*-5a gives rise to spatially restricted transcripts. In contrast, *Wnt*-5b was apparently transcribed uniformly. To verify the *Wnt*-5 transcription patterns seen by in situ hybridization, we performed Northern blot analysis of *Wnt*-5 transcripts in microdissected regions of the fetus. RNA was collected from the face, brain, and limb at 10.5 and 14.5 days (Fig. 8). At 10.5 days, dissection of the forelimb into distal and proximal halves revealed considerably higher levels of *Wnt*-5a transcripts in the distal half, as expected. In contrast, approximately similar low levels of *Wnt*-5b transcripts were detected in both fractions. At this time the hindlimb is too small to dissect into proximal and distal fractions. However, elevated levels of *Wnt*-5a were present in the hindlimb fraction relative to the RNA fraction derived from the total fetus (Fig. 8). By 14.5 days higher levels of *Wnt*-5a were readily apparent in the distal fraction of fore- and hindlimbs and, as earlier, no difference in the levels of *Wnt*-5b was detected (Fig. 8). At 10.5 days, higher levels of *Wnt*-5a were detected in the brain and face relative to the total fetus; but as the sites of accumulation of *Wnt*-5a transcripts represented only a small fraction of each sample, the differences detected at this time by Northern analysis were not dramatic (Fig. 8). In contrast, by 14.5 days, most of the frontal region of the face expressed high levels of *Wnt*-5a. As expected, *Wnt*–5a transcripts were significantly enriched in this fraction (Fig. 8). As in all other fractions, only low constitutive expression of *Wnt*-5b was seen (Fig. 8). Hybridization of the Northern blots with a β-actin probe demonstrated that the above results were not due to variations in the amount of RNA loaded. Moreover, as the β-actin-coding probe cross-hybridizes with muscle actin transcripts, under these conditions, this probe provides an indication of the state of differentiation of limb tissues in the 14.5-day fractions. The actin probe detects a lower muscle-specific transcript in only the proximal limb fraction, which is relatively more differentiated than the distal fraction.

Adult expression of the Wnt *genes*

Expression of *Wnt*-1 and *Wnt*-2 is not restricted to fetal

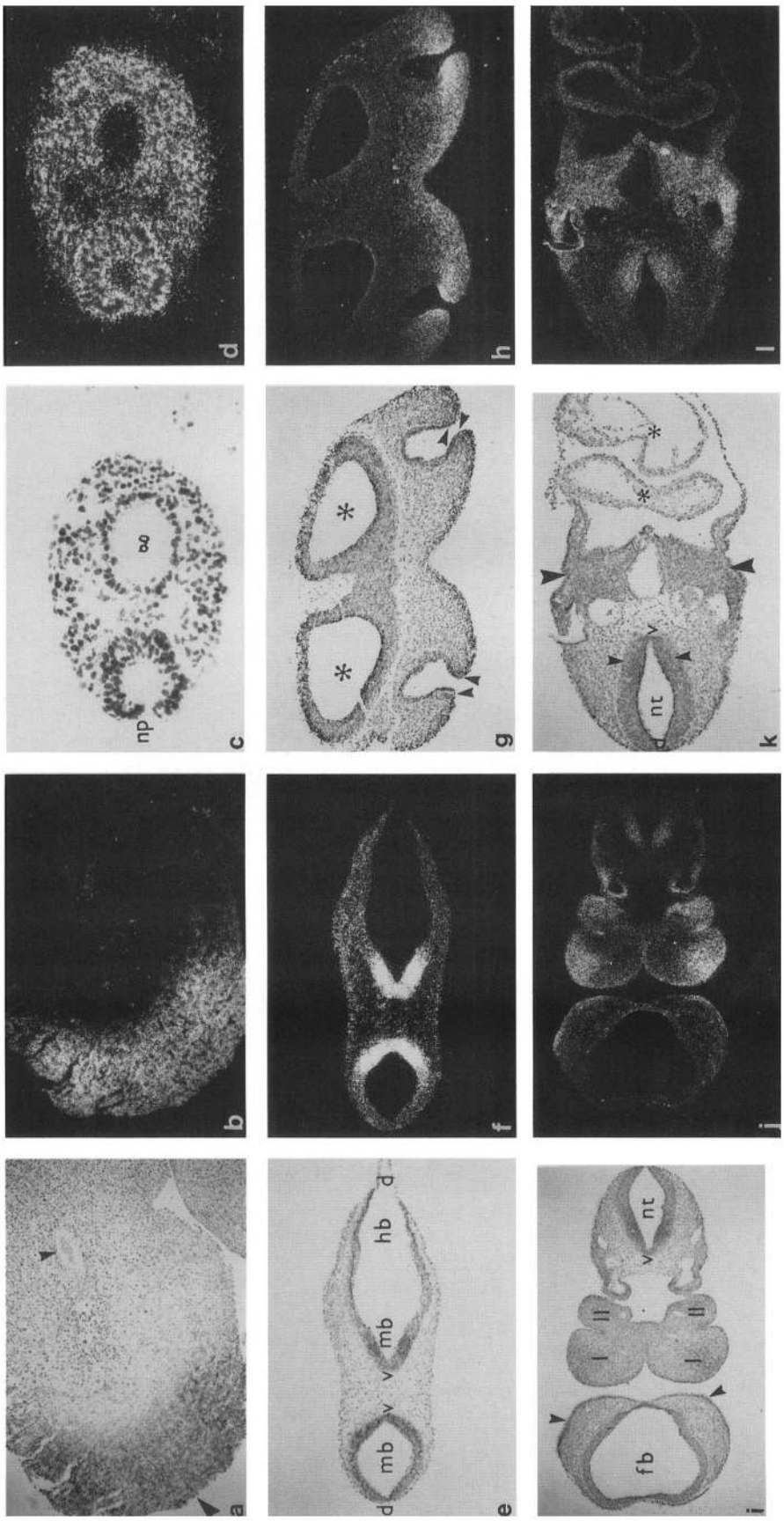

Figure 6. Expression of *Wnt*-5a in the developing conceptus. (*a,b*) Section through a 6.5-day decidual mass. High levels of *Wnt*-5a transcripts are localized at the mesometrial pole of the deciduum (large arrowhead). The position of the embryo is indicated by the small arrowhead. (*c,d*) Transverse section through the open posterior neuropore (np) of a 9.5-day mouse fetus. *Wnt*-5a transcripts are distributed throughout the neuroectoderm, the mesoderm, and the endodermal epithelium of the gut (g). (*e,f*) Coronal section through the midbrain (mb) and hindbrain (hb) of a 9.5-day mouse fetus. Expression is seen in the ventral (v) but not the dorsal (d) midbrain. (*g,h*) Transverse section through the developing frontal–nasal processes of a 10.5-day fetus. *Wnt*-5a transcripts are located toward the tips of the processes (small arrowheads). Expression occurs in both the neural crest-derived mesenchyme and in the ectoderm surrounding this region but is not seen in more distal neural crest or ectoderm, nor in the neuroepithethelium encompassing the telencephalic vesicles (asterisks). (*i,j*) Transverse section through the forebrain (fb), I and II branchial bars (I, II), and rostral spinal cord (nt) of a 9.5-day mouse fetus. *Wnt*-5a transcripts are localized in the facial epithelium (small arrowheads), distally in the branchial bars and in the ventral (v) region of the spinal cord, excluding the extreme ventral floor plate. (*k,l*) Transverse section through heart (*), lateral mesoderm (large arrowheads), and spinal cord (nt) of a 9.5-day mouse fetus. *Wnt*-5a transcripts are present in the lateral mesoderm and in the ventral (v) neural plate (small arrowheads) but are excluded from the floor plate. (*a,c,e,g,i,k*) Bright-field micrographs; (*b,d,f,h,j,l*) dark-field micrographs.

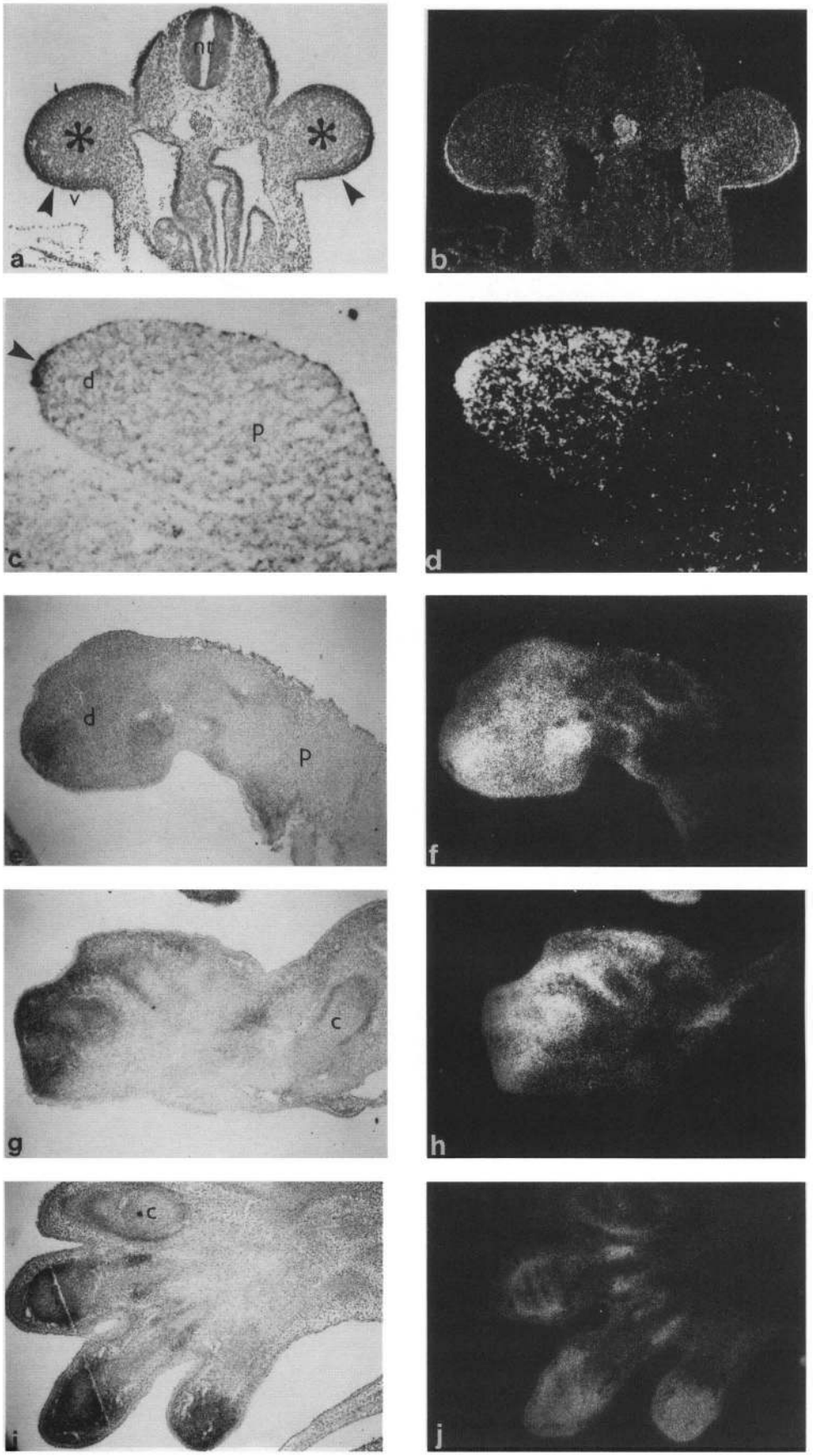

Figure 7. *(See facing page for legend.)*

life; specific adult sites of expression have also been noted. *Wnt*-1 is only expressed in round spermatids of the adult testes (Shackleford and Varmus 1987), and *Wnt*-2, in heart and lung (McMahon and McMahon 1989). Therefore, these genes presumably act quite differently in adult and fetal stages. RNA blot analysis of the expression of the six new genes in a variety of adult tissues indicates distinct expression profiles. All six genes are expressed in adult brain, although *Wnt*-5a, *Wnt*-5b, and *Wnt*-6 transcripts are present at very low levels (Fig. 9). In addition, all but *Wnt*-6 were also expressed in adult lung. *Wnt*-4 and *Wnt*-7a were only expressed in brain and lung. *Wnt*-7b and *Wnt*-5a were also expressed in a third tissue, kidney, and heart, respectively (Fig. 9). Like *Wnt*-1, *Wnt*-6 is expressed at high levels in the adult testes (Fig. 9). In contrast to the other members, *Wnt*-5b was widely expressed in all tissues analyzed with the exception of adult spleen. All of the new members show extremely low levels of expression in the undifferentiated CCE embryo stem cell line (Fig. 9).

It is apparent from this analysis that although tissue distributions show some overlap, particularly in brain and lung, there are also tissues in which expression is limited to only one family member. Moreover, members of the two closely related pairs, *Wnt*-5a and *Wnt*-5b, and *Wnt*-7a and *Wnt*-7b, show distinct distributions. *Wnt*-5b is expressed more widely and at higher levels in adult tissue than *Wnt*-5a. At first sight, *Wnt*-7a and *Wnt*-7b appear to be more similar. Not only do they give rise to transcripts of nearly identical size (4.4 kb), but they also share restricted expression patterns. However, on closer analysis of brain fractions (Fig. 10), expression is clearly distinct. *Wnt*-7a is expressed at high levels in the cerebellum and at lower levels in cerebrum, diencephalic–mesencephalic, and myelencephalic fractions (Fig. 10). In contrast, *Wnt*-7b shows complementary expression, elevated in the latter three fractions, but present at only low levels in the cerebellum. Thus, expression of even closely related family members differs substantially.

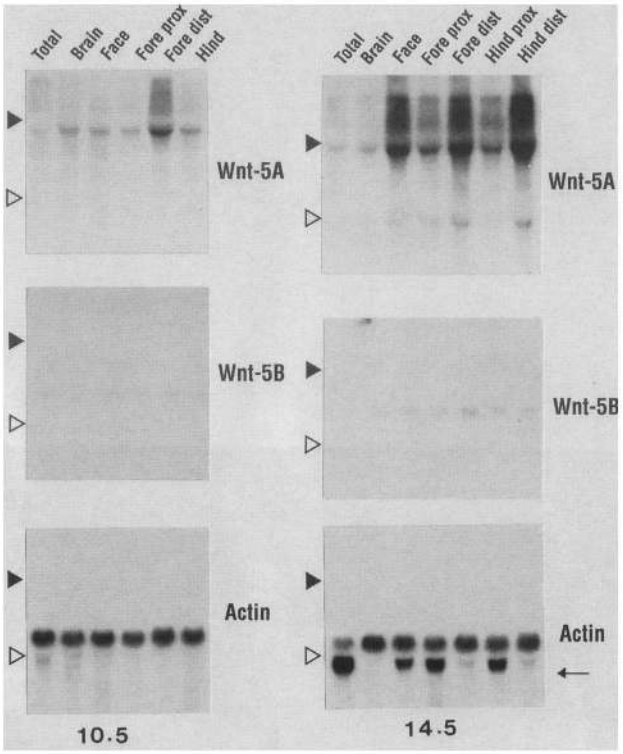

Figure 8. Expression of *Wnt*-5a and *Wnt*-5b in tissue fractions from 10.5- and 14.5-day fetuses. At 10.5 days *Wnt*-5a is elevated clearly in the distal forelimb and shows slightly elevated levels of expression in whole-brain, face, and hindlimb fractions relative to RNA from the total fetus. By 14.5 days, high levels of *Wnt*-5a transcripts are clearly present in distal fore- and hindlimb fractions corresponding to the developing hand plate. The developing bone region makes up the proximal fraction. In addition, high levels of *Wnt*-5a transcripts are seen in the face. In contrast, only low, uniform levels of *Wnt*-5b RNA were detected in all fractions after long exposure (2 weeks) of Northern blots. Hybridization of the *Wnt*-5a Northern blots with a cytoplasmic β-actin probe demonstrates that equivalent amounts of RNA were loaded in all lanes. The position of the muscle-specific actin band, which occurs in the proximal limb, face, and total embryo RNA samples, is indicated by the arrow. Solid arrowheads indicate 28S rRNA; open arrowheads 18S rRNA.

Discussion

Using a PCR-based strategy, we have identified six new members of the *Wnt* family that are expressed during fetal development. Like *Wnt*-1 and *Wnt*-2, the new related proteins have a hydrophobic amino terminus and a number of absolutely conserved amino acid residues, most notably 21 cysteines. The functional importance of a single cysteine substitution (McMahon and Moon 1989a) suggests that these play a critical role, presumably in inter- or intramolecular cross-linking of the proteins. By analogy with *Wnt*-1, it seems reasonable to propose that these new members are secreted and participate in cell signaling events.

All of the *Wnt*-1-related genes are expressed over a broad period of fetal development. At the earliest stages, the processes of gastrulation, neurulation, somitogenesis, and organogenesis generate and organize the germ layers and organ anlagen. By the end of fetal devel-

Figure 7. *Wnt*-5a expression in the developing forelimb bud. (*a*,*b*) Initial expression at 9.5 days is restricted to the ventral (v) ectoderm (arrowheads) of each forelimb bud (*). Note at this level that no neural tube (nt)-associated expression of *Wnt*-5a is visible. (*c*,*d*) At 10.0 days, expression is localized to the distal apical ectoderm and underlying mesenchyme (d) but is not seen in proximal (p) mesenchyme. At 11.5 (*e*,*f*), 12.5 (*g*,*h*), and 14.5 days (*i*,*j*) most expression remains distally localized, but is restricted to mesenchyme at these times. Some expression is also seen surrounding condensing cartilaginous elements (c), but no expression is detected in differentiating cartilage or bone. Planes of sections are transverse sections along the anterior to posterior axis (*a*–*d*); horizontal sections along the dorsal–ventral axis (*e*–*j*). (*a*,*c*,*e*,*g*,*i*) Bright-field micrograph; (*b*,*d*,*f*,*h*,*j*) dark-field micrographs.

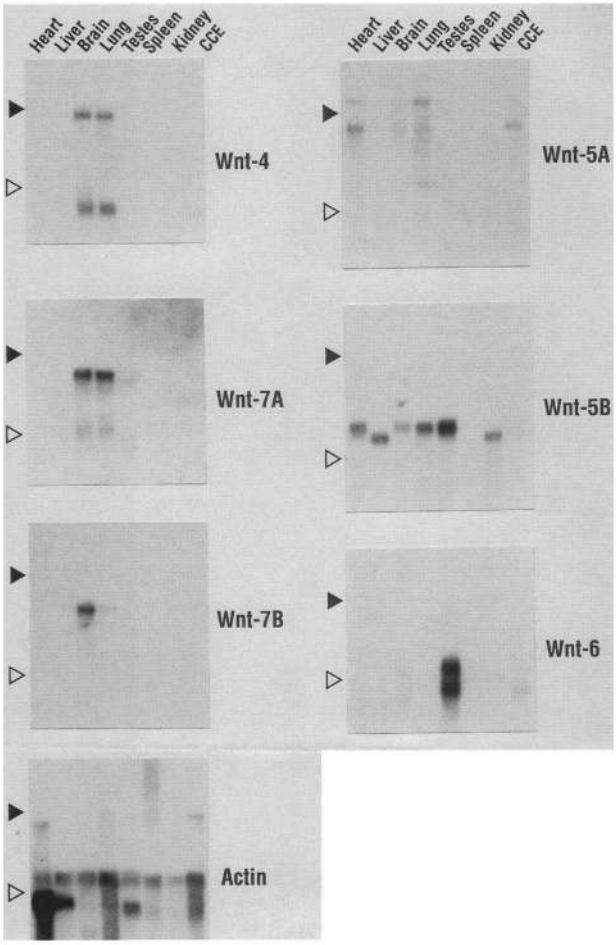

Figure 9. Expression of novel *Wnt*-1-related genes in adult tissues. The indicated adult tissue and CCE embryo stem cell RNA (Robertson et al. 1986) were analyzed by Northern blot hybridization for expression. In addition, filters were probed with a β-actin probe to verify equal loading and integrity of RNAs. The hybridizing actin band just above the 18S rRNA position represents cytoplasmic β-actin transcripts; the lower band represents muscle-specific actin transcripts. Solid and open arrowheads are positions of 28S and 18S rRNAs, respectively.

opment, most adult structures are well differentiated, the major changes that occur after birth being largely restricted to the nervous and reproductive systems. Thus, it is unlikely that the various signaling events that initially required the participation of these genes in early fetal life are also operative shortly before birth. Rather, the continued expression throughout fetal life of the *Wnt* genes probably reflects the acquisition of new roles as development proceeds. Confirmation of this view will require detailed knowledge of spatial expression and protein function but is supported by initial studies. We have studied in detail the expression of two closely related genes, *Wnt*-5a and *Wnt*-5b. Whereas transcripts for *Wnt*-5b show no apparent spatial or temporal restriction but are widely distributed, *Wnt*-5a shows several distinct sites of accumulation. Several of these sites, including the CNS, the limb, the facial processes, and the posterior region of the fetus, correlate with spatial patterning and morphogenesis. However, the diverse nature of the different sites that express *Wnt*-5a suggests that *Wnt*-5a, like other secreted peptide factors including *int*-2 (Wilkinson et al. 1988, 1989) and members of the transforming growth factor-β (TGF-β) family (Lyons et al. 1989), probably performs many different functions, depending on the context in which it is expressed. Initial studies on other members of this family suggest that multiple sites of expression is a general rule, to which *Wnt*-1 is the major exception (J.A. McMahon and A.P. McMahon, unpubl.). Clearly, the mutational strategy that has proved so valuable in the analysis of *Wnt*-1 will be necessary for establishing the essential functions of *Wnt*-5a and the other *Wnt* genes.

Redundant functions for the Wnt *family?*

From the data presented here and previous studies on *Wnt*-1 (Jakobovits et al. 1986; Wilkinson et al. 1987a), *Wnt*-2 (McMahon and McMahon 1989), and *Wnt*-3 (Roelink et al. 1990), it is clear that even at the earliest stages of fetal development nine related genes are expressed, although expression has only been demonstrated at the RNA level thus far. It is possible that all nine genes may be involved in different regulatory pathways; alternatively, there might be redundancy in the action of family members. That some functional redundancy exists is suggested by the phenotype of mice homozygous for *Wnt*-1 *null* alleles (McMahon and Bradley 1990; Thomas and Capecchi 1990), in which the hindbrain and spinal cord, which are both normal sites of expression of *Wnt*-1, develop apparently normally. However, in situ hybridization of *Wnt*-1 (Wilkinson et al. 1987) and *Wnt*-2 (McMahon and McMahon 1989) indicates that there is no overlap in expression of these members. As discussed, *Wnt*-1 is localized exclusively to the neural tube and *Wnt*-2 to the pericardium, the ventral lateral mesoderm, and the allantois and its derivatives. Thus, *Wnt*-1 and *Wnt*-2 show distinct patterns of spatial expression. While the detailed spatial analysis of expression of all the genes reported here has yet to be determined, at least *Wnt*-5a has patterns of expression that are largely nonoverlapping with those of *Wnt*-1 and *Wnt*-2. For example, *Wnt*-5a is expressed in the ventral

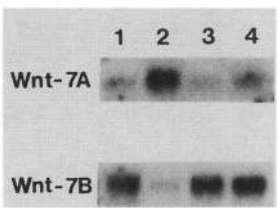

Figure 10. Expression of *Wnt*-7a and *Wnt*-7b in total RNA from adult brain fractions. Brains were dissected into four fractions: cerebrum (lane *1*); cerebellum (lane *2*); diencephalon–mesencephalon (lane *3*); mylencephalon (lane *4*). RNAs were isolated and examined for expression by Northern blot analysis.

midbrain at 9.5 days, when most *Wnt*-1 expression in this region is localized dorsally. Moreover, *Wnt*-5a shows extensive expression in the face, limbs, and posterior region of the fetus, none of which express *Wnt*-1 or *Wnt*-2. In contrast, both *Wnt*-2 and *Wnt*-5a are expressed in lateral mesodermal populations, although careful serial section analysis will be required to determine whether these populations overlap. Furthermore, it is clear that *Wnt*-5a and *Wnt*-5b show quite distinct expression profiles, suggesting different functional roles for this pair of highly related genes. In general, these observations do not support the view of extensive functional redundancy in the developmental roles of the *Wnt* family of proteins, although some redundancy probably does exist. Rather, they suggest that although the presumed pathway of action is similar—secreted factor, interacting with cell surface receptor, triggering signal transduction pathway(s)—the actual developmental processes regulated by their action are different for each protein.

This view of nonredundancy is substantiated further by examination of adult expression. Quantitative and qualitative differences are seen in the expression profiles of all *Wnt*-1-related genes, although in the lung and brain at least a low level expression of most of this family is observed. Of particular relevance is expression of the more closely related genes, *Wnt*-5a and *Wnt*-5b, and *Wnt*-7a and *Wnt*-7b. As in the fetus, *Wnt*-5a and *Wnt*-5b show distinct differences in the tissue specificity of expression. Similarly, although *Wnt*-7a and *Wnt*-7b are both expressed at moderate levels in the adult brain, a closer inspection revealed different spatial patterns of expression. Therefore, although some redundancy may exist in the activity of *Wnt* genes in the fetus and adult, this is probably the exception and not the rule.

How many Wnt *genes?*

The strategy we have adopted to search for *Wnt*-1-related genes has a number of biases. The 5′ oligonucleotide set included degenerate primers for all codons except the CA$^{C}_{T}$ 3′-histidine codon. Only the CAT codon was present at this position. Thus, priming on a cDNA template in which histidine is now encoded by CAC leads to a mismatch at the start of new DNA synthesis, which has been shown to affect amplification adversely (Kumar 1989). However, it should be noted that these primers amplified *Wnt*-1 and *Wnt*-4, both of which utilize a CAC histidine codon. More importantly, the initial screen was aimed specifically at early fetal stages to deliberately clone only those *Wnt*-1-related genes that might function at this time. Thus, screening genomic DNA might identify new genes. Finally, examination of a larger number of PCR products might also identify new members.

Recently, Roelink et al. (1990) have identified a new MMTV integration site, *Wnt*-3, implicated in mammary tumorigenesis, which encodes a *Wnt*-1-related gene different from those discussed here. As *Wnt*-3 is expressed during fetal life, our inability to isolate *Wnt*-3 indicates clearly that other related genes may also have been missed. Recently, we have attempted to isolate new clones using 9.5-day cDNA and primers utilizing the CAC codon for histidine. To date, we have isolated clones for *Wnt*-1, *Wnt*-6, *Wnt*-4, and *Wnt*-3, but no new members (of 39 *Wnt* clones analyzed; J.A. McMahon and A.P. McMahon, unpubl.). Thus, although it is impossible to predict accurately the number of family members, in light of the current identification of nine members the real figure probably lies somewhere between 10 and 20.

From PCR analysis of other species, it is clear that multiple *Wnt*-1-related genes are not confined to the mouse. Related sequences have been isolated from *Xenopus* (Christian et al. 1991), *Drosophila* (A. McMahon, in prep.), and *C. elegans* (Kamb et al. 1989). Thus, in both vertebrate and invertebrate species, there are likely to be multiple roles for the *Wnt*-1 family.

Wnt-*1-receptors, mammary tumors, and axial duplication*

Although there is no solid evidence for its existence, the normal interaction of peptide-signaling molecules with a cell surface receptor suggests that *Wnt* action is also mediated in this way. In *Drosophila*, mutants that resemble *wingless* (*Drosophila Wnt*-1) but act cell-autonomously, are likely receptor candidates. As yet, no mutants of this type have been molecularly identified. The demonstration of multiple signaling molecules related to *Wnt*-1 suggests that either a single receptor is capable of interacting with a number of proteins with limited amino acid homology (50–60%) or that a divergent receptor family exists. Two lines of evidence suggest that in abnormal situations, different *Wnt*-1-related proteins may interact in the same receptor pathway, although this in no way implies that it happens normally in vivo.

Wnt-1 and *Wnt*-3, when activated by MMTV insertion, give rise to mammary tumors (for review, see Nüsse 1988). However, in normal mammary glands there is no evidence for expression of *Wnt*-1 or *Wnt*-3 (Nüsse et al. 1984; Wilkinson et al. 1987a; Roelink et al. 1990). Thus, a receptor is presumably present and able to respond to either of these proteins, but does not normally do so. Preliminary evidence indicates that several of the new *Wnt* clones are expressed in the normal mammary gland and their expression is regulated during pregnancy and lactation (B. Gavin and A. McMahon, unpubl.). Therefore, the abnormal action of *Wnt*-1 and *Wnt*-3 may be mediated by interaction with a receptor normally present to receive a signal from one or more of the related *Wnt* proteins that normally regulates mammary gland development.

Similarly, ectopic expression of mouse *Wnt*-1 in *Xenopus* embryos leads to duplication of axial mesodermal tissue (McMahon and Moon 1989b), implying a specific, active receptor pathway well before normal *Xenopus Wnt*-1 expression (Noordemeer et al. 1989). Thus, this receptor pathway may normally respond to

another *Wnt* protein, the deregulated expression of *Wnt*-1 perturbing this interaction. In support of this idea, evidence exists for the early localized expression of a *Xenopus Wnt*-1-related gene that can interact with this same pathway (Christian at al. 1990). Clearly, if these suggestions are correct, misexpression of other family members in these systems might be expected to result in similar outcomes. This result is not surprising, given the paradigm of the fibroblast growth factor (FGF) family. The mesoderm-inducing properties of many of the FGF family members strongly suggest that this family of peptide growth factors can also "cross talk" in FGF receptor-mediated pathways (Paterno et al. 1989).

In summary, the evidence presented here supports the view that the *Wnt* family has a major role in development of the mouse. The *Wnt* family, along with those of TGF-β and FGF, constitutes three superfamilies of putative signaling molecules that presumably act to regulate developmental processes. The determination of the multiple developmental roles of the *Wnt* family members will present a major challenge.

Materials and methods

PCR amplification and cloning of Wnt*-1-related genes from 9.5-day fetal cDNA*

Total RNA was isolated from 9.5-day mouse fetuses using the lithium chloride/urea procedure (Auffray and Rougeon 1980). First-strand cDNA was synthesized from 10 μg of total RNA using oligo(dT) priming with an Invitrogen kit in 50 μl volume, as recommended by the manufacturer. cDNA was amplified using 200 ng of each of the following degenerate oligonucleotide primers (see Fig. 1A):

GGGGAATTCCA(C/A) GA(A/C) TG(C/T) AA(A/C) TG(T/C) CAT (5′ primer) and
AAAATCTAGA(C/A) CA(C/A) CACCA(C/A) TG(A/C) AA (3′primer).

Standard PCR amplification used one-fortieth of the cDNA reaction or 1 ng of control *Wnt*-1- or *Wnt*-2-containing plasmids in a final volume of 20 μl of 50 mM KCl, 10 mM Tris-HCl (pH 8.4) 1.5 mM $MgCl_2$, 20 μg/ml gelatin, 0.2 mM dNTPs (Pharmacia), and 0.6 units *Taq* polymerase (Perkin-Elmer/Cetus). Reactions were overlaid with 25 μl of mineral oil and amplified using a thermal cycler (M-J Research) as follows: 2 min at 94°C, then 30 cycles of 90 sec at 55°C, 1 min at 72°C, 1 min at 94°C, followed by 30 min at 37°C. Five microliters of the reaction products were separated in a 2% agarose gel, the remainder was brought up to 100 μl with water, extracted once with aqueous phenol and once with Sevag, and precipitated by the addition of one-tenth volume of 3 M sodium acetate and two volumes of 100% ethanol. Following recovery of ethanol precipitates, amplified DNA was digested to completion with 20 units each of *Eco*RI and *Xba*I and subcloned into *Eco*RI/*Xba*I-cleaved pGEM 3Zf (Promega). Recombinant clones were identified by *Eco*RI/*Xba*I digestion of alkaline lysis-prepared minipreps (Maniatis et al. 1982) and double-stranded sequenced using a T7 polymerase sequencing kit (Pharmacia) according to the manufacturer's instructions.

Isolation and sequencing of Wnt*-1-related λ cDNA clones*

PCR-generated fragments for *Wnt* clones 6, 7A, 5A, and 61 were oligolabeled with [α-^{32}P]dCTP using a random priming kit (Multiprime, Amersham). Replica filter lifts of ~6 × 10^5 recombinants of an 8.5-day mouse cDNA library (Fahrner et al. 1987) were hybridized with 5 × 10^5 cpm/ml of each probe. Hybridizations were performed overnight at 42°C in 50% formamide, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 0.05 M Tris-HCl (pH 7.5), 1.0 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, and 100 μg/ml yeast RNA. Filters were washed 3 × 15 min at room temperature in 2× SSC, 0.2% SDS and 3 × 30 min at 68°C in 0.2× SSC, 0.1% SDS. On the final round of screening, quadruplicate plaque lifts were screened individually with each probe. Individual clones were extended 5′ and 3′ by repeated screening of up to 5 × 10^6 plaques of the λgt10 library. Inserts were cloned into the *Eco*RI site of pGEM7Z (Promega) following PCR amplification and *Eco*RI digestion of phage λ isolates or following Lambdasorb (Promega) isolation and *Eco*RI digestion of recombinant phage.

To obtain clones encoding the extreme 5′ sequence of *Wnt*-6 and *Wnt*-7a, 10^6 clones from a P20 mouse whole-brain Uni-Zap XR cDNA library (Stratagene) were screened with extreme 5′ probes. Methods were as detailed above with the exception that recombinant plasmids were excised from recombinant phage using R408 helper phage, as recommended by the vendor (Stratagene).

All cDNA inserts were sequenced on both strands using a combination of specific oligonucleotide primers and directed subcloning. Sequencing was performed using double-stranded dideoxy methods with a T7 polymerase sequencing kit, as recommended by the manufacturer (Pharmacia).

Isolation of Wnt*-7b and* Wnt*-4 genomic DNA*

Genomic *Wnt*-7b DNA encoding the ambiguous cysteine residue (see Results) was amplified using previously described conditions and the following oligonucleotides: GGGGAATTCCGGGCGTGGCGCATGCTGTC (5′ primer) and AAAATCTAGAGGCATCCACAAAGCGACGAG (3′ primer). Restriction digests, subcloning, and sequencing were exactly as detailed earlier.

A *Sau*3A λ DASH (Stratagene) mouse genomic library (the generous gift of Ross Kinloch) was screened with an oligolabeled *Wnt*-4 cDNA probe, as detailed previously. Mouse genomic DNA was isolated, *Bam*HI-digested, and blotted to nitrocellulose, and genomic fragments surrounding splice acceptor and donor sites were identified by hybridization with oligonucleotides (Maniatis et al. 1982). The appropriate *Bam*HI fragments were subcloned into *Bam*HI-cut pGEM7Z and sequenced.

RNA blot analysis

Total RNA from fetal or adult tissues was prepared by either the LiCl/urea (Auffray and Rougeon 1980) or the guanidinium hydrochloride (Maniatis et al. 1982) procedure. Poly(A) RNA was isolated following a single round of oligo(dT) selection using an Invitrogen Fast Trak mRNA isolation kit according to the manufacturer's instructions. Alternatively, poly(A) RNA was isolated directly from lysed tissues using the same kit.

Approximately 2.5 μg of isolated poly(A) RNA was separated electrophoretically on a 1.2% formaldehyde agarose gel (Maniatis et al. 1982), transferred to nylon membrane (GeneScreen, Dupont), UV cross-linked (Church and Gilbert 1984), and hybridized with 1.5 × 10^6 cpm/ml of random-primed (Amersham) [^{32}P]dCTP-labeled β-actin (Minty et al. 1981) or *Wnt* cDNA probes. For the adult brain fractions and microdissected embryo fractions, 5 μg of total RNA was used for each sample. Hybrid-

izations were at 45°C in 50% formamide, 0.25 M sodium phosphate (pH 7), 0.25 M NaCl, 7% SDS, 1 mM EDTA, and 10% PEG-20,000. Washes were as follows: 3 × 20 min at room temperature in 2× SSC and 0.2% SDS, 2 × 30 min at 68°C in 0.2× SSC and 0.2% SDS, and 2 × 1 hr in 1× SSC, and 0.2% SDS at 65°C. All autoradiography was performed using intensifying screens at −70°C.

In situ hybridization

In situ hybridization was performed essentially as described by Wilkinson et al. (1987b). *Wnt*-5a and *Wnt*-5b probes used for in situ hybridization were identical to those used on Northern analysis. These studies, together with Southern blot analysis, indicate no cross-reactivity under the condition used.

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B J Gavin, J A McMahon and A P McMahon

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