Isolation and Characterization of a Mouse Homolog of the *Drosophila* Segment Polarity Gene *dishevelled*

DANIEL J. SUSSMAN,[*,1,2] JOHN KLINGENSMITH,[†‡1] PATRICIA SALINAS,[†] PAMELA S. ADAMS,[§]
ROEL NUSSE,[†] AND NORBERT PERRIMON[‡]

Division of Human Genetics, Department of Obstetrics and Gynecology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, Maryland 21201-1559; †Howard Hughes Medical Institute, Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305; ‡Howard Hughes Medical Institute, Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115; and §The W. Alton Jones Cell Science Center, Lake Placid, New York 12946

In the *Drosophila* embryo *dishevelled* (*dsh*) function is required by target cells in order to respond to *wingless* (*wg*, the homolog of *Wnt-1*), demonstrating a role for *dsh* in *Wnt* signal transduction. We have isolated a mouse homolog of the *Drosophila dsh* segment polarity gene. The 695-amino-acid protein encoded by the mouse *dishevelled* gene (*Dvl-1*) shares 50% identity (65% similarity) with *dsh*. Similarity searches of protein and DNA data bases revealed that *Dvl-1* encodes an otherwise novel polypeptide. While no functional motifs were identified, one region of *Dvl-1* was found to be similar to a domain of *discs large-1* (*dlg*), a *Drosophila* tumor suppressor gene. In the embryo, *Dvl-1* is expressed in most tissues, with uniformly high levels in the central nervous system. From 7.5 days postcoitum *Dvl-1* is expressed throughout the developing brain and spinal cord, including those regions expressing *Wnt-1* and *En*. Expression of *Dvl-1* in adult mice was found to be widespread, with brain and testis exhibiting the highest levels. The majority of *Dvl-1* expression in the adult cerebellum is in the granular cell layer, similar to the pattern seen for *engrailed-2* (*En-2*). Throughout postnatal development of the brain *Dvl-1* is highly expressed in areas of high neuronal cell density. © 1994 Academic Press, Inc.

INTRODUCTION

The isolation and characterization of mouse homologs of *Drosophila* genes has been a fruitful approach for the study of mammalian development. For example, the gene order of the *antennapedia*-related homeobox-containing genes has been conserved through evolution. Cloning of the mammalian HOX homologs has led to the identification of a group of genes which function in the

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[1] The first two authors contributed equally and amicably to this work.
[2] To whom correspondence should be addressed.

establishment of the body plans of vertebrate embryos (for review see Krumlauf, 1993).

Characterization of mouse homologs of the *Drosophila* segment polarity genes should likewise prove very valuable, in that these genes mediate cell–cell interactions which result in cell fate determination during embryonic and adult fly development. Structural and functional evidence indicates that many segment polarity genes are involved in intercellular communication (reviewed by Klingensmith and Perrimon, 1991; Perrimon, 1994).

The best-characterized murine segment polarity gene homologs are *Wnt-1* and *En-2*. The *Wnt-1* (*int-1*) proto-oncogene, identified and isolated by virtue of its activation by Mouse Mammary Tumor Virus insertion, is the homolog of the *wingless* (*wg*) segment polarity gene (Rijsewijk *et al.*, 1987). *Wnt-1* is a member of a family of related genes that are differentially expressed during development and appear to be involved in cell signaling (reviewed by Nusse and Varmus, 1992; McMahon, 1992). *Wnt-1* encodes a cysteine-rich secreted protein that remains associated with the extracellular matrix. The expression of *Wnt-1* in mammary tissue of transgenic mice results in benign hyperplasia and subsequent adenocarcinomas. The *Wnt-1* gene is normally expressed between Days 8.5 and 14 during embryogenesis and expression is restricted to specific regions of the developing neural tube (Wilkinson *et al.*, 1987; Bally-Cuif *et al.*, 1992). The phenotype of mice homozygous for a *Wnt-1* null allele, generated by homologous recombination, is dramatic in that some mice are born with virtually no cerebellum and no midbrain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). The *swaying* mutation, characterized by ataxia, hypertonia, and malformation of the anterior cerebellum, was shown to be due to a point mutation in the *Wnt-1* gene (Thomas *et al.*, 1991).

Two mouse genes homologous to *engrailed* (*En-1*, Joyner *et al.*, 1985; and *En-2*, Joyner and Martin, 1987)

have been cloned. The *En* genes encode putative transcription factors and are initially coordinately expressed with *Wnt-1* during the definition of spatial domains in the developing central nervous system (Davis and Joyner, 1988; McMahon *et al.*, 1992). By mid-embryogenesis the expression patterns of *Wnt-1* and the two *En* genes themselves diverge markedly, becoming highly restricted as development progresses. For example, only *En-2* is expressed in the granule cell layer of the cerebellum (Davis and Joyner, 1988; Davis *et al.*, 1991). Mice homozygous for the targeted deletion of the *En-2* homeobox have a smaller cerebellum than wildtype animals, displaying abnormal foliation (Joyner *et al.*, 1991). In contrast, *En-1* mutants have a deletion of structures at the midbrain–hindbrain junction apparent by 9.5 days of development, very similar to that of *Wnt-1* mutants (Wurst *et al.*, 1994).

In *Drosophila* embryos *wg* and *en* are expressed in adjacent, non-overlapping stripes of cells yet have a mutually dependent requirement for maintenance of expression (Martinez-Arias *et al.*, 1988; DiNardo *et al.*, 1988). Thus, *wg* expression decays in *en* mutants and *en* expression decays in *wg* mutants. These effects must be mediated by signaling between these domains of cells. In the case of the *wg* signaling pathway, the signal is most likely encoded by *wg* itself (reviewed by Klingensmith and Nusse, 1994). McMahon *et al.* (1992) have reported that in the mouse embryonic midbrain, maintenance of *En* expression requires *Wnt-1* function, suggesting possible conservation of the *wg-en* pathway in mouse development.

The *Drosophila* segment polarity gene *dishevelled* (*dsh*) has an embryonic phenotype indistinguishable from *wg* (Perrimon and Mahowald, 1987; Klingensmith and Perrimon, 1991), suggesting participation in the same developmental processes. *dsh* also functions in *wg*-mediated axial patterning of legs and wings in adults (Klingensmith *et al.*, 1994). In segmentation, *dsh* is required for the maintenance of *en* expression by the adjacent *wg*-secreting cells (van den Heuval *et al.*, 1993). *dsh* also mediates the post-transcriptional effects of *wg* on *armadillo* protein, occurring in the *wg*-expressing cells as well as flanking cells (Riggleman *et al.*, 1990). Genetic epistasis studies indicate that *dsh* functions downstream of the *wg* signal (Noordermeer *et al.*, 1994). Moreover, the requirement for *dsh* function is cell-autonomous, meaning that each cell must make *dsh* to achieve its appropriate fate (Klingensmith *et al.*, 1994). These results indicate that *dsh* is involved in reception or transduction of the *wg* signal in target cells.

We have utilized a *Drosophila dsh* cDNA probe to isolate a mouse homolog (*Dvl-1*). We find that *Dvl-1* is expressed throughout the embryonic central nervous system from presomite stages and in neuron-rich areas of the brain throughout postnatal development, as well as

in many other tissues. The realm of *Dvl-1* expression includes those tissues and stages in which *Wnt-1* and *En* genes are expressed.

MATERIALS AND METHODS

Library Screens and Hybridizations

The *Drosophila dsh* probe used was a 0.65-kb *Pst*I to *Xho*I fragment from the middle of the coding region of dc2.6 (Klingensmith *et al.*, 1994). To determine whether *dsh* sequences were conserved in other species, approximately 10 µg of genomic DNAs from the species indicated in Fig. 1 were digested with *Eco*RI and electrophoresed on a 0.8% agarose gel. Fragments were transferred to Gene Screen (NEN Research Products). Hybridization was performed with the ^{32}P-labeled *dsh* probe at 42°C overnight in the buffer described below. The filter was washed twice for 30 min at room temperature in 2× SSC/0.1% SDS, followed by two washes for 30 min at 65°C in 1× SSC/0.1% SDS. The autoradiograph was exposed for 5 days.

The *dsh* probe was also used to screen a mouse Balb C genomic DNA λ Fix II library (a generous gift from Dr. Laurie Jackson-Grusby). A mouse brain cDNA λ Zap library was purchased from Stratagene and a mouse Day 11.5 embryo cDNA λ GT10 library was purchased from Clontech. Libraries were plated in top agarose at a density of 3 to 5 × 10^5 PFU per 24 × 24-cm plate. Plaques were transferred to duplicate membranes of Gene Screen Plus (Dupont/NEN). Membranes were allowed to dry for 10 min, layered between Whatman 3-mm chromatography paper, and then, to burst the phage, sterilized in an autoclave for 2 min, followed by fast exhaust for 2 min. Membranes were incubated for 1 hr at 42°C with shaking in prewash solution (50 mM Tris, pH 8, 1 M NaCl, 1 mM EDTA, 0.1% SDS). Membranes were incubated for 2 hr at 42°C with shaking in prehybridization solution (10% dextran sulfate, 40% formamide, 4× SSC, 1× Denhardt's, 7 mM Tris, pH 7.6, 20 µg/ml sheared denatured salmon sperm DNA, and 0.1% SDS). DNA probes were labeled with [^{32}P]dCTP using the random hexamer method (Boehringer-Mannheim Biochemicals) and added to prehybridization solution at 10^6 cpm/ ml. Hybridization was performed for 12 hr at 42°C. Low-stringency wash of the membranes was performed using 1× SSC/0.1% SDS at room temperature for 30 min (one change of wash) and 1× SSC/0.1% SDS for 60 min (one change of wash) at 65°C. High-stringency wash was the same as low-stringency except that the final wash was performed using 0.1× SSC/0.1% SDS.

DNA Sequencing and Analysis

DNA sequencing (dideoxy method) was performed using both [^{35}S]dATP manual (United States Biochemical

Sequenase Version 2.0) and automated (Applied Biosystems Model 370A Sequencer) techniques. Sequencing of both strands was accomplished by generating subclones utilizing the Promega Erase-a-Base System. Regions that were missed or ambiguous were sequenced utilizing oligonucleotide primers generated on an Applied Biosystems Model 391 DNA Synthesizer. The DNA sequence of Dvl-1 cDNA is present in the GenBank database under Accession No. U10115.

Sequence similarity searching of the GenBank data base (version 72) and structural analysis was done using software (IFIND, BLAST, and QUEST) from Intelligenetics, Inc. The PEST-FIND software was a generous gift from Dr. Martin Rechsteiner.

RNA Expression Analysis

For ribonuclease (RNase) protection assays, total RNA was isolated according to the procedure of Chirgwin et al. (1979). RNase protection assays were performed according to the procedure described by Melton et al. (1984). A 269-bp MBG5 *NaeI/BglII* fragment was subcloned into pBlueScript KS to generate riboprobes. Subclone MBG5-269 was digested with *XhoI* and transcribed *in vitro* in the presence of [^{32}P]UTP using T7 polymerase (Boehringer-Mannheim Biochemicals). The ribosomal protein L32 riboprobe, used as an internal standard, was a generous gift of Dr. Michael M. Shen.

The substrate for reverse transcription–polymerase chain reaction (RT–PCR) was poly(A)$^+$ RNA, isolated from whole embryos or adult brains via the "Quickprep micro" mRNA purification kit (Pharmacia); 200 ng RNA was reverse transcribed with 10 units/microliter MMLV reverse transcriptase (Gibco-BRL), primed by 5 μM random hexanucleotides (Boehringer-Mannheim). Reaction conditions were as recommended by the enzyme manufacturer. PCR amplification used primers from the 3' untranslated region of *Dvl-1*. Dvl1.s1 began at position 2973 in the sequence as follows: 5'-TACTGAAGGTAT-GTGAAG-3'. Dvl1.a1 was the reverse complement of a stretch of bases ending at position 3175, as follows: 5'-TGTCTAGTGTAGATGTGT-3'. Amplification of 1/10 of each cDNA reaction in the presence of 1.5 mm MgCl$_2$ was performed by *Taq* polymerase from Promega according to manufacturer's recommendations on a PTC-100 thermocycler (MJ Research). The cycling parameters were as follows: 94° 30 sec, 46° 40 sec, 72° 30 sec, repeated 40 times, followed by a final extension of 5 min at 72°. Amplification products were electrophoresed on a 3% Nuseive, 1% agarose (FMC) gel.

In Situ Hybridization

Strand-specific sense and antisense RNA probes were synthesized using T7 and T3 RNA polymerases (Boehringer-Mannheim and Stratagene) and labeled by incor-poration of [^{35}S]UTP (Amersham) using the conditions of Melton et al. (1984). The principal template used was a 877-bp fragment of MBG5 (bases 1213 to 2089) obtained by PCR (utilizing oligonucleotide primers DJS69, 5'-TGTACGGGTGCTTCGGGAG-3' and SAP4, 5'-ACT-TGGTACTGTGTGGTCTG-3') and subcloned into the *SmaI* site of pKS. These probes were degraded to an average length of 500 bases by alkaline hydrolysis (Cox et al., 1984). A second, non-overlapping template was a 367-bp *KpnI* to *PstI* fragment from the 3' noncoding region of MBG5. Before use *in situ*, these probes were used in RNase protection assays to make sure only the expected *Dvl-1* transcripts were detected.

Embryos and brains were obtained from CD1 mice and processed and sectioned as described by Roelink and Nusse (1991). *In situ* hybridizations were based on the procedure of Wilkinson et al. (1987) as modified by Salinas and Nusse (in preparation). To summarize, probes were diluted to 7 × 10^4 cpm/μl in hybridization buffer and applied to the sections, which were hybridized for 16 hr at 60°C. Slides were washed in 2× SSC/50% formamide/0.14% β-mercaptoethanol at 65°C for 30 min and then transferred to 37°C for 60 min. RNase A digestion (20 μg/ml) of unhybridized transcript was performed in NTE (0.5 M NaCl/10 mM Tris, pH 8.0/5 mM EDTA) for 30 min at 37°C. The final washes were in 2× SSC/50% formamide for 30 min at 37°C and at room temperature in 0.1× SSC. After dehydration through ethanol and air drying, slides were covered with Kodak NTB2 emulsion and allowed to expose for 10 days at 4°C. After development embryos and brain sections were counterstained in methyl green. Analysis was principally via bright- and dark-field microscopy, and Nomarski optics in some cases, on a Nikon Microphot microscope.

Whole-mount *in situ* hybridization followed exactly the protocol of Conlon and Rossant (1992). In addition to *Dvl-1* sense and antisense RNA probes, antisense probes for spatially restricted messages were also used as positive controls. For the sectioned material, these included *Wnt-3* and *Wnt-3a* (Roelink and Nusse, 1991) and brachyury (Wilkinson et al., 1990) for whole-mount experiments.

RESULTS

Isolation of Mouse Sequences Homologous to dishevelled

Prior to screening a mouse genomic library, we wished to determine if a *Drosophila dsh* cDNA probe could hybridize to sequences in genomic DNA obtained from a number of vertebrate organisms. Figure 1 shows an autoradiograph of a Southern blot hybridized to a *dsh* cDNA probe and washed at low stringency. Sequences were detected by the *dsh* probe in all the species tested, including mouse; this suggests that *dsh* may be conserved broadly among vertebrates.
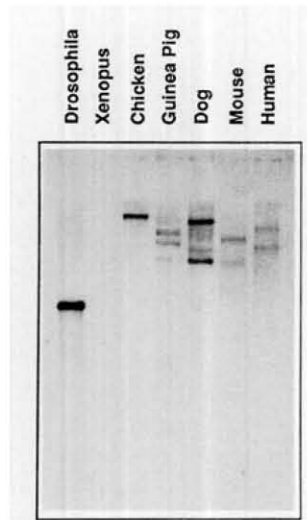
FIG. 1. Southern blot analysis of genomic DNAs using a *Drosophila dsh* cDNA probe. Genomic DNAs of the species indicated were digested with restriction enzymes, resolved by agarose gel electrophoresis, and transferred to a nylon membrane. The filter was hybridized at low stringency with a *dsh* cDNA probe derived from the coding region. The hybridizing *Drosophila* genomic fragment (4.6 kb) serves as a positive control.

The fly *dsh* probe was then used to screen a Balb C mouse genomic λ Fix II library (see Materials and Methods). DNA was isolated from positive plaques, digested with a number of restriction endonucleases, and analyzed by Southern blot using the *dsh* probe (data not shown). Subclones of *dsh*-positive restriction fragments were obtained and sequenced. A subclone of 1 kb in length, X6sac, contained considerable amino acid sequence similarity to *dsh* arranged in the form of 6 exons and 6 introns, all of which are about 100 bp in length (Nardos Lijam and D.J.S., unpublished data).

Utilizing a portion of the X6sac subclone for ribonuclease protection analysis, we detected relatively high levels of expression in mouse embryo and brain RNA samples (data not shown). The X6sac subclone was used to screen mouse brain and mouse Day 11.5 embryo cDNA libraries (see Materials and Methods). Several positive plaques were isolated from each library. The cloned positive cDNAs were sequenced as described under Materials and Methods. The open reading frame derived from mouse brain cDNA clone MBG5 is compared to that of *Drosophila dsh* (Klingensmith *et al.*, 1994) in Fig. 2. There is 50% sequence identity (65% similarity) between the *dsh* and MBG5 coding regions. An embryo-derived cDNA clone, d11.5C2, is identical to MBG5, except for a 75-bp deletion that removes 25 amino acids without altering the reading frame. Ribonuclease protection analysis of RNA isolated from a number of tissues as well as embryos, revealed that the d11.5C2 deletion is not present at detectable levels over the full-length protected fragment; thus, d11.5C2 is not a major

alternately spliced species (data not shown). The DNA sequence and restriction map of the putative full-length cDNA clone MBG5 are shown in Fig. 3.

Dvl-1 Sequence Analysis

The open reading frame encoded by MBG5, *Dvl-1*, consists of 695 amino acids with a calculated molecular weight of 75,312 Da. While there are no inframe termination codons upstream of the *Dvl-1* assigned initiation methionine, this initiation site was chosen on the basis of its Kozak consensus sequence (Kozak, 1986) and its position with respect to *dsh* sequence similarity. This protein does not appear to contain any signal sequence and/or a significant membrane-spanning potential. A comparison of *dsh* and *Dvl-1* reveals similar hydropathy profiles, extending over both conserved and nonconserved regions with respect to amino acid sequence (data not shown). The *Dvl-1* encoded protein contains three potential N-linked glycosylation sites, one of which is conserved, and many potential kinase sites. Both proteins appear to have PEST-like sequences, associated with rapid turnover of enzymes, transcription factors, and components of signal transduction pathways (Rogers *et al.*, 1986; Rechsteiner, 1990). The *Dvl-1* encoded protein contains three possible PEST sequences, with the highest PEST-FIND score being 13.1 for amino acids 175 to 209. The PEST-FIND score, which can range from −45 to +50, is based on the mole percent of proline (P), glutamic acid (E), aspartic acid (D), serine (S), and threonine (T), as well as hydrophilicity. The PEST-FIND scores obtained for *Dvl-1* are in the same range as those obtained for proteins with a half-life of 0.5 hr. The *Dvl-1* encoded protein contains two RGD tripeptide sequences, a motif which is associated with recognition systems for cell surface signaling (Ruoslahti and Pierschbacher, 1986); however, these sequences are not present in the *dsh* encoded protein. Searches of GenBank and EMBL data bases with the *Dvl-1* encoded protein sequence revealed no overall sequence similarity to other known genes.

Dvl-1 and dsh Encoded Proteins Exhibit Similarity to Segments of lethal(1)discs large-1 (dlg)

A search of the GenBank database for amino acid sequence similarity to *Dvl-1* reveals a stretch of homology to apparently repeated segments of the *dlg* gene, a *Drosophila* tumor suppressor gene (Woods and Bryant, 1991). One of these repeats corresponds to the "undefined domain" of dlg and p55 (Bryant and Woods, 1992), which has not yet been associated with a specific function. Klingensmith *et al.* (1994) reported the alignment of *dlg* with *Dvl-1* and *dsh*. Interestingly, this 48-amino-acid region of *Dvl-1*, spanning amino acids 277 to 324, displays 88% identity with the corresponding region of the *dsh* protein.

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dsh   1   MDADRGGGQETKVIYHIDDETTPYLVKIPIPSAQVTLRDFKLVL.NKQNNNYKYFFKSMD  59
            ||| ||| | | |||||| |       ||| ||| || |    || ||||||
dvl   1     MAETKIIYHMDEEETPYLVKLPVAPERVTLADFKNVLSNRPVHAYKFFFKSMD  53


dsh  60   ADFGVVKEEIADDSTILPCFNGRVVSWLVSADGTNQSDNCSELPTSECELGMGLTNR.KL 118
          || |||||| ||   ||||||||||||| | |  ||     |     |     |
dvl  54   QDFGVVKEEIFDDNAKLPCFNGRVVSWLVLAEGA.HSDAGSQGTDSHTDLPPPLERTGGI 112


dsh 119   QQQQQQHQQQQQQQQQQHQQQQQQQQQQVQPVQLAQQQQQQVLHHQKMMGNPLLQPP..P 175
                                      |
dvl 113   GDSRPPSFEPNVASSRDGMDNETGTESMVSHRRERARRRNRDEAARTNGHPRGDRRRDLG 172
                              *


dsh 176   LTYQSAS.VLSSDLDSTSLFGTESELTLDR..DMTDYSSVQRLQVRK.KPQRRKKRAPSM 231
          | ||| |||| | |          |  |     || ||| |    ||||
dvl 173   LPPDSASTVLSSELESSSFIDSDEEDNTSRLSSSTEQSTSSRL.VRKHKCRRRKQRLRQT 231
                                    *


dsh 232   SRTSSSRSITDSTMSLNIITVSINMEAVNFLGISIVGQSNRGGDGGIYVGSIMKGGAVAL 291
          | ||  ||||||||||||| |||  ||| |  |||||||||| |||||| |||||||||||
dvl 232   DRASSFSSITDSTMSLNIITVTLNMERHHFLGISIVGQSNDRGDGGIYIGSIMKGGAVAA 291


dsh 292   DGRIEPGDMILQVNDVNFENMTNDEAVRVLREVVQKPGPIKLVVAKCWDPNPKGYFTIPR 351
          |||||||| ||||||||||||| || ||||||  |||   ||| |||||||||  ||||||
dvl 292   DGRIEPGDMLLQVNDVNFENMSNDDAVRVLREIVSQTGPISLTVAKCWDPTPRSYFTIPR 351
                             *


dsh 352   TEPVRPIDPGAWVAHTQALT....................SHDSIIADIAEPIKERLD. 389
          ||||||| ||  || |||               |      | |
dvl 352   ADPVRPIDPAAWLSHTAALTGALPRYGTSPCSSAITRTSSSLTSSVPGAPQLEEAPLTV 411


dsh 390   QNNLEEIVKAMTKPDSGLEIRDRMWLKITIPNAFIGADAVNWVLENVEDVQDRREARRIV 449
          ||  | ||||||||||||||||||||||||| || |||| | |  || |||||
dvl 412   KSDMSAIVRVMQLPDSGLEIRDRMWLKITIANAVIGADVVDWLYTHVEGFKERREARKYA 471


dsh 450   SAMLRSNYIKHTVNKLTFSEQCYYVVNEERNPNLLGRGHLHPHQLPHGHGGHALSHADTE 509
          | || |||||||||||||||||   ||     | |   ||       |    | ||
dvl 472   SSMLKHGFLRHTVNKITFSEQCYYVF.GDLCSNLAS........LNLNSGSSGASDQDT. 521


dsh 510   SITSDIGPLPNPPIYMPYSATY.....NPSHGYQPIQYGIAERHISSGSSSSDVLTSKDT 564
           ||| | |   | |    |     |      |      |     | || |      |
dvl 522   .....LAPLPHPSVPWPLGQGYPYQYPGPPPCFPP...AYQDPGFSCGSGSAGSQQSEGS 573


dsh 565   SASQSDITSVIHQANQLTIAAHGSNKSSGSSNRGGGGGGGGGGGNNTNDQDVSVSNYVL  622
          |                          | ||| |            |
dvl 574   KSSG.....................STRSSHRTPGREERRATGAGGSGSESDHTVPSGS  611


dvl 612   GSTGWWERPVSQLSRGSSPRSQASAVAPGLPPLHPLTKAYAVVGGPPGGPPVRELAAVPP  671


dvl 672   ELTGSRQSFQKAMGNPCEFFVDIM                                      695
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FIG. 2. Comparison of *dsh* and *Dvl-1* encoded proteins. The open reading frames derived from *dsh* and *Dvl-1* are aligned with sequence identity represented by the vertical lines. The underlined region (solid line) corresponds to the amino acids absent from the embryo-derived *Dvl-1* cDNA clone d11.5C2. The three potential PEST sequences are underlined with a dotted line. The three potential N-linked glycosylation sites are indicated with an asterisk.

Characterization of Dvl-1 Expression by RNase Protection and RT–PCR Analysis

To characterize *Dvl-1* gene expression, we analyzed a number of adult mouse tissues, cell lines, and Day 9.5 to 11.5 embryos by RNase protection assay. An RNase protection probe was generated from a *NaeI/BglII* subclone of MBG5, which protects a 269-base region of 5' *Dvl-1* transcript. Included in each assay as an internal standard is a riboprobe that protects the ribosomal L32

protein transcript. As shown in Fig. 4A, the tissues with the highest levels of *Dvl-1* expression are testis, brain, and kidney. Lower levels of expression were detected in ovary, breast, muscle, liver, and small intestine, with spleen and thymus exhibiting the lowest levels. A moderate level of *Dvl-1* expression was detected in adult heart (data not shown). Figure 4B reveals that expression of *Dvl-1* is detected in Day 9.5 embryo RNA and increases approximately 2.5-fold by Day 11.5. Expression of *Dvl-1* was found to be low in NIH 3T3 and Balb C 3T3
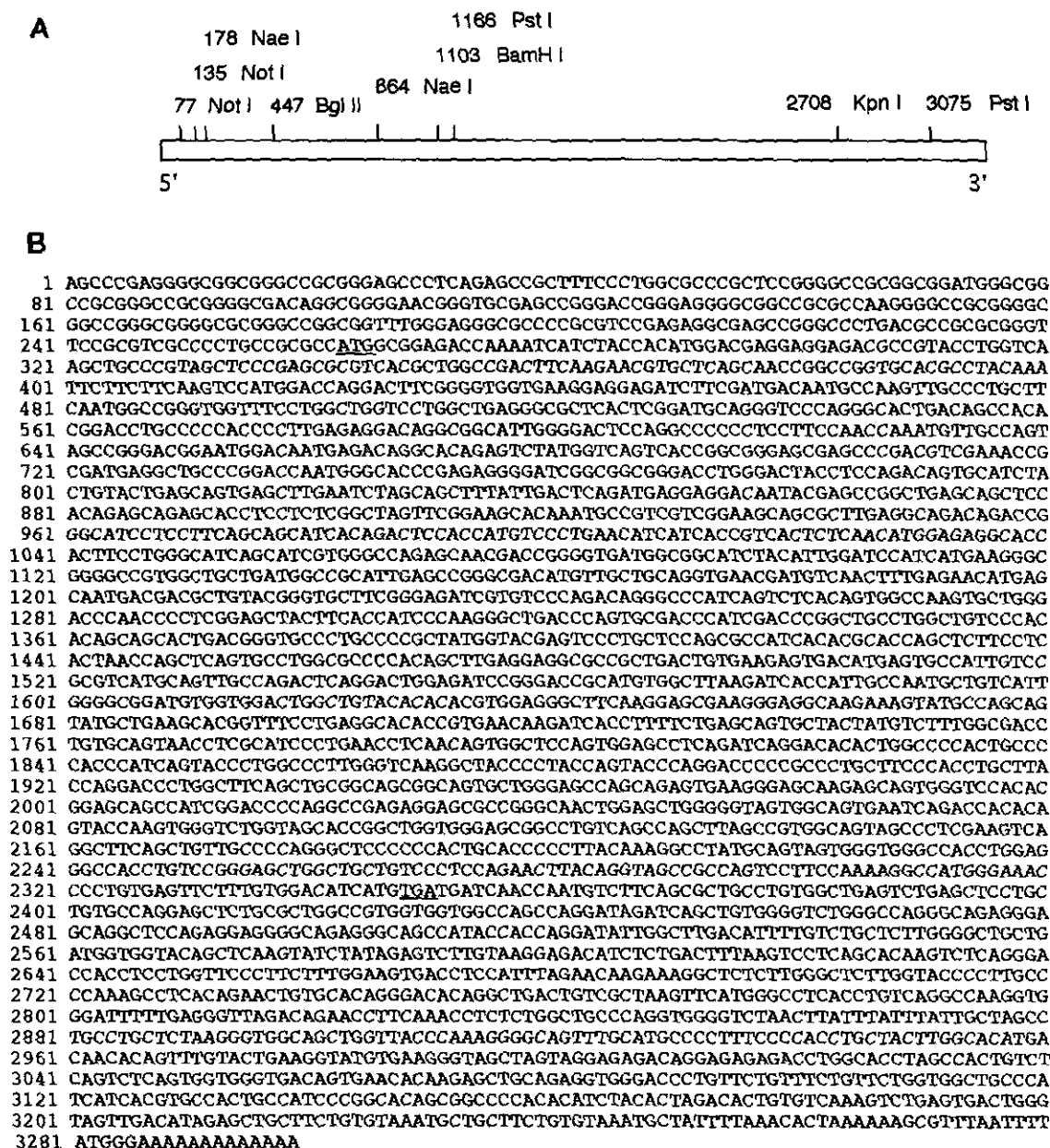
FIG. 3. Restriction map and nucleotide sequence of clone MBG5. (A) Restriction map of *Dvl-1* mouse brain cDNA clone MBG5. The map displays several restriction endonuclease sites relevant to the subclones utilized for RNase protection and *in situ* hybridization analyses. (B) Nucleotide sequence of *Dvl-1* mouse brain cDNA clone MBG5. The codons representing the initiation methionine and translation termination are underlined.

cell lines and was also detected in RNA obtained from mouse embryonic stem cells (data not shown).

We confirmed and extended these findings to earlier embryonic stages via amplification of *Dvl-1* messages by the polymerase chain reaction from cDNA reverse transcribed from RNA purified at various embryonic stages. A single band was amplified from each stage, corresponding to the predicted size based on the product amplified from the cDNA clone MBG5 (Fig. 4C). Thus, *Dvl-1* is expressed significantly from at least 7.5 days of development, rising to high levels by midgestation. It continues to be expressed

in adults, occurring in a wide variety of tissue types derived from all three primary germ layers.

Characterization of Dvl-1 Expression by in Situ Hybridization

We investigated the spatial pattern of *Dvl-1* expression during embryogenesis to address its role in development. Because in *Drosophila* *dsh* mediates signaling between *Wnt-* and *en*-expressing cells, while in targeted mouse mutants both *Wnt-1* and *En-1* are required for
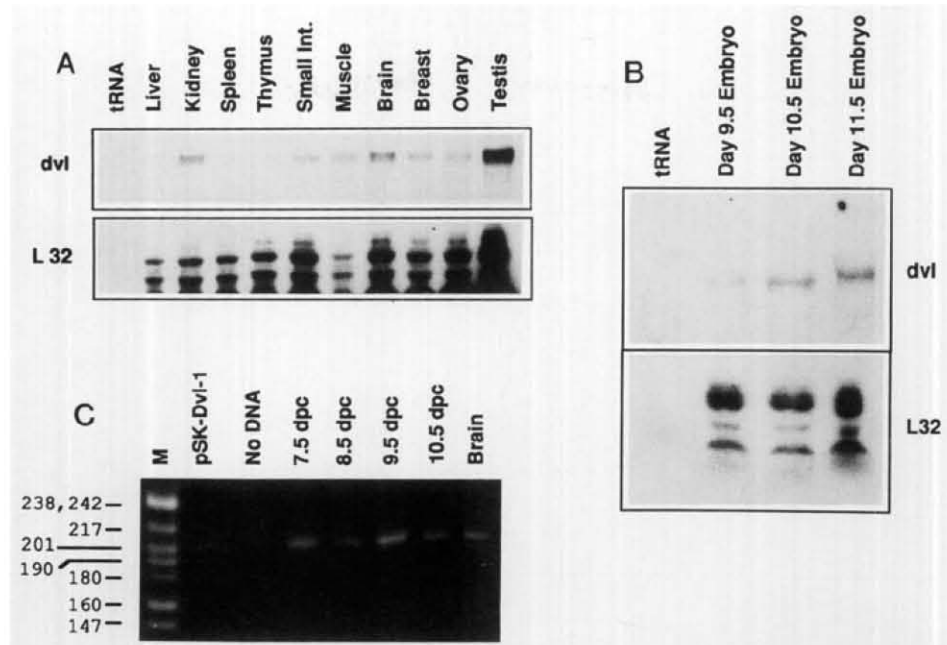
FIG. 4. Analysis of *Dvl-1* gene expression by RNase protection and RT–PCR. (A, B) Total RNA (20 μg) was analyzed by ribonuclease protection using a riboprobe (MBG5 *NaeI/BglII* fragment subcloned into pBlueScript KS) designed to protect a 269-base fragment of *Dvl-1* RNA. Included in each assay is an internal standard riboprobe that protects 83 bases of ribosomal L32 protein transcript. The adult tissue or developmental stage (in days postcoitum) of each sample is indicated above the corresponding lane. (C) At the stages indicated, mRNA was harvested and reverse transcribed with random priming and then subjected to PCR with *Dvl-1* primers designed to amplify a 202-bp amplicon. In addition, 5 ng MBG5 (*Dvl-1*) cDNA was subjected to PCR as a positive control. For comparison, a sample of RNA from adult brain was also prepared. In each case a single band was amplified, of approximately 202 bp compared to size marker bands (left). A sample containing water only was amplified as a negative control, which showed no bands at any size range.

brain patterning, we were especially interested in *Dvl-1* expression in the central nervous system (CNS). Nonisotopically labeled strand-specific probes were hybridized to whole embryos aged 7.5, 8.5, and 9.5 days postcoitum (d.p.c.), reflecting a continuous developmental range of late primitive streak through the 25 plus somite stage (Fig. 5). Single-stranded radiolabeled antisense and sense probes from two non-overlapping fragments of the cDNA MBG5 were hybridized *in situ* to sections from wildtype embryos 8.5, 9.5, 10.5, 12.5, 15.5 and 16.5 d.p.c., providing a range of specimens from early somitogenesis through late organogenesis (Fig. 6). Positive controls on the sectioned and whole-mount embryos were performed using *Wnt-3* (Roelink and Nusse, 1991) and *brachyury* (Wilkinson *et al.*, 1990), respectively (data not shown).

Dvl-1 expression is widespread during embryogenesis, with high levels occurring in the CNS at all time points examined. Embryos at the headfold and early somite stages (7.5–8.0 d.p.c.) show expression throughout the neural folds and in most mesodermal tissues (Fig. 5B). However, *Dvl-1* does not appear to be expressed in the node (Fig. 5C), the source of key pattern-organizing signals (Beddington, 1994). In embryos 8.5 d.p.c., by which time many somites have formed but the neural tube has not yet closed, *Dvl-1* is expressed at low levels through-

out the CNS and most other tissues (Fig. 6B). Transcripts are expressed at higher levels in the extraembryonic membranes surrounding the fetus. By 9.0 days expression is higher, occurring intensely in the spinal cord and throughout the developing brain (Fig. 6D), as well as in many other tissues. Expression of *Dvl-1* at 10.5 d.p.c. remains at high levels in much of the embryo (Fig. 6F). At 12.5 d.p.c. *Dvl-1* expression is still abundant in many developing tissues (e.g., liver, kidney, intestine, lung, tongue) but is highest throughout the CNS and most or all major ganglia (Figs. 7 and 5D). By 15.0 d.p.c. expression throughout the embryo has decreased, occurring at lower levels in the CNS and in many tissues of the embryo (data not shown).

As indicated by RNase protection, expression of *Dvl-1* in the adult brain is very high. To further characterize *Dvl-1* expression by *in situ* hybridization, we sectioned brains in various planes from postpartum animals aged 0, 1, 6, 15, 22, 28, and 60 days. Expression of *Dvl-1* occurs at high levels in several parts of the brain from birth to adulthood. Most notably, there are high levels of transcription in the olfactory bulb (Fig. 8A) in the stratum pyrimidale and stratum granulosum of the hippocampal formation (Fig. 8B) and in the granular cell layer of the cerebellum (Fig. 8C). All of these areas are very dense with neuronal cell bodies. Isolated areas of high expres-
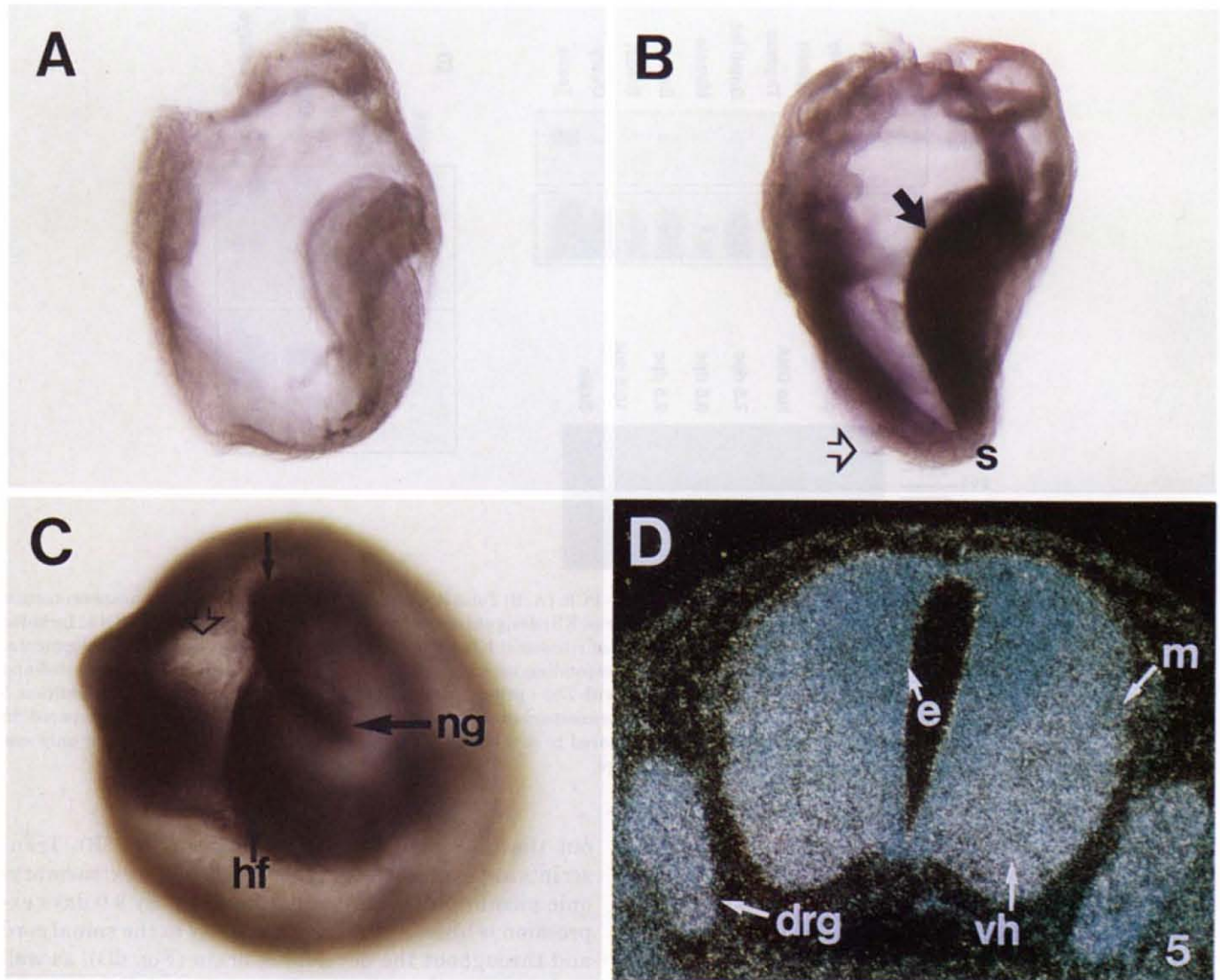
FIG. 5. Whole-mount *in situ* hybridization and transverse spinal cord section. Embryos hybridized with *Dvl-1* probes. (A and B) Whole-mount 7.5 d.p.c. embryos at the initiation of somitogenesis. Anterior is to the left and dorsal is up. (A) Sense probe. (B) Antisense probe reveals that *Dvl-1* expression is widespread, especially strong throughout the neural folds. The presumptive midbrain is indicated by a black arrowhead. *Dvl-1* is expressed in the mesoderm, including the one to two somites(s) but not in the node (open arrowhead). (C) Ventral view of the embryo shown in (B). The absence of expression in the node is indicated by the open arrowhead. Expression in the headfolds (hf) is shown by small arrows and in the neural groove (ng) by a larger arrow. (D) Embryonic Day 12.5 transverse section hybridized to *Dvl-1* antisense probe (dorsal is uppermost). Expression is seen at high levels in spinal cord, including the ventral horns (vh), the postmitotic mantle (m), and proliferative ependymal layers (e). Expression of *Dvl-1* is also high in the dorsal root ganglia (drg).

FIG. 8. Expression of *Dvl-1* in the postnatal brain. Saggital sections of brains dissected from mice of the indicated ages were hybridized with *Dvl-1* antisense probes. Adjacent sections hybridized with *Dvl-1* sense probe showed very low background (data not shown). (A) Adult olfactory bulb section. Expression occurs in the granule cell layer (GL), the mitral layer (ML), and in the periglomerular cells (PC). (B) Adult hippocampal formation section. High levels of *Dvl-1* expression are detected in the stratum pyrimidale (SP) and the stratum granulosum (SG) of the dentate gyrus. (C) Adult cerebellum section. Very high expression of *Dvl-1* is seen in the granular cell layer (GCL) of neurons and glia. (D) Postnatal Day 1 (P1) brain section. The GCL of the cerebellum is formed via inward migration of pluripotent cells from the external germinal layer (EGL). *Dvl-1* expression is detected in both layers at this stage. (E) P6 brain section. The expression of *Dvl-1* has broadened, reflecting the expansion of both GCL and EGL. Expression is also seen in a multilayered pattern in the cerebral cortex (CC) and in the hippocampal formation (HF). (F) P15 brain section. At P15 the EGL is shrinking as cells migrate through the expanding molecular layer (ML) to form the broadening GCL. Expression of *Dvl-1* in the hippocampal formation (HF) is indicated. (G) P22 brain section. The EGL disappears by the end of the third postnatal week, all cells having migrated inward. At P22, no hybridization is detected outside the molecular layer (ML), being restricted to the GCL.
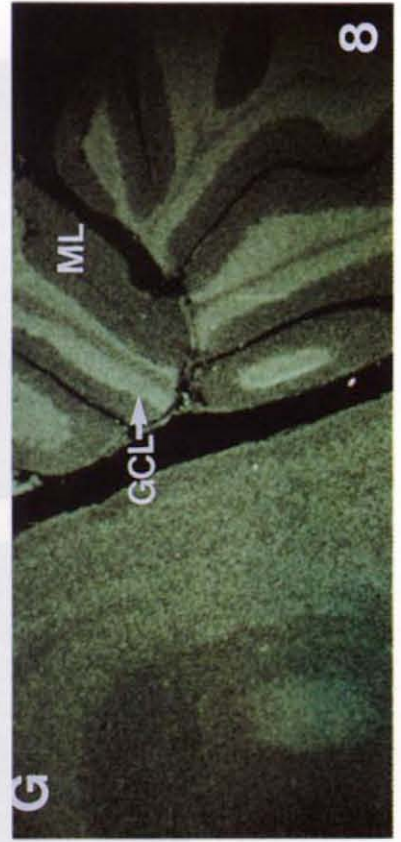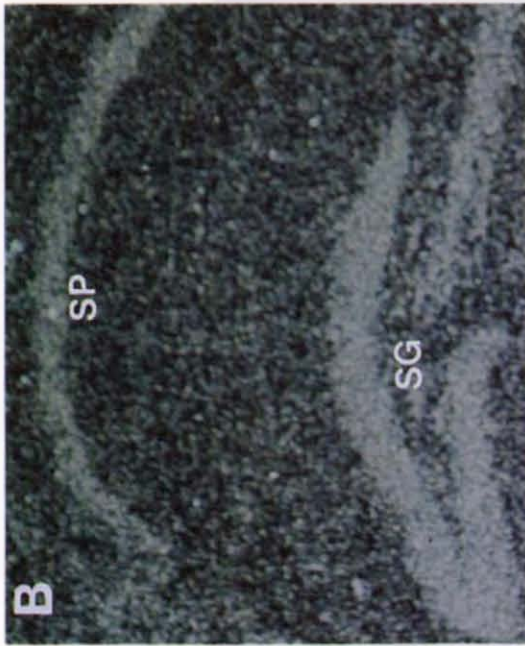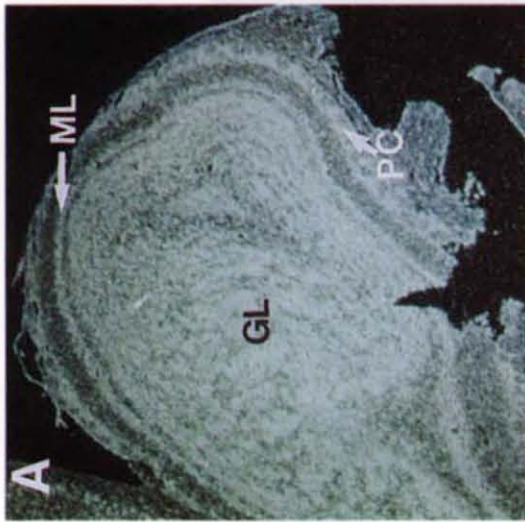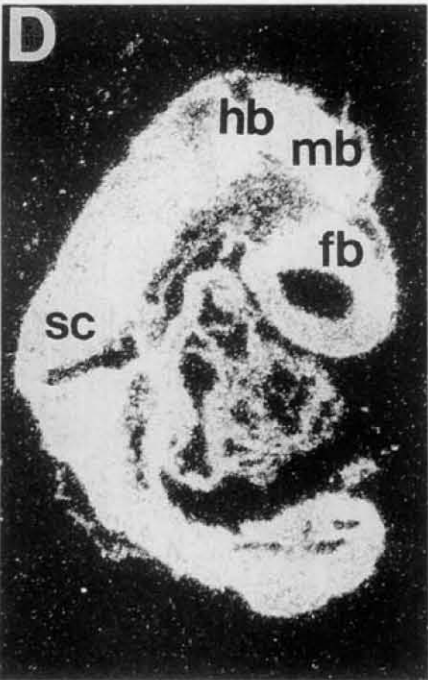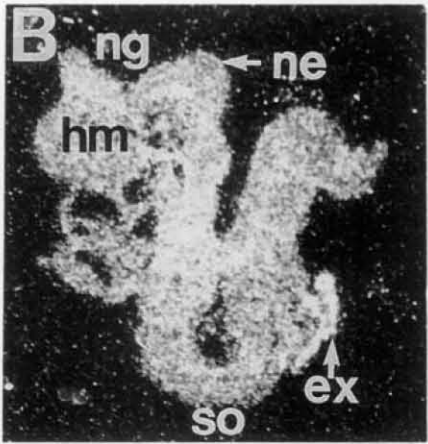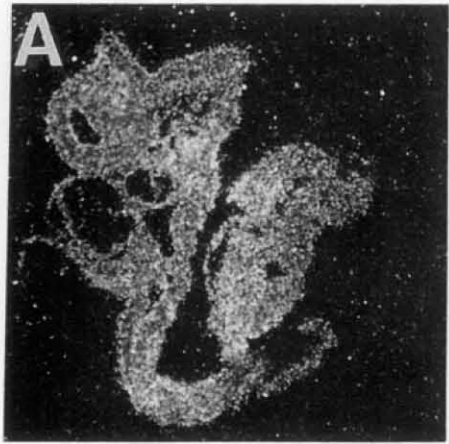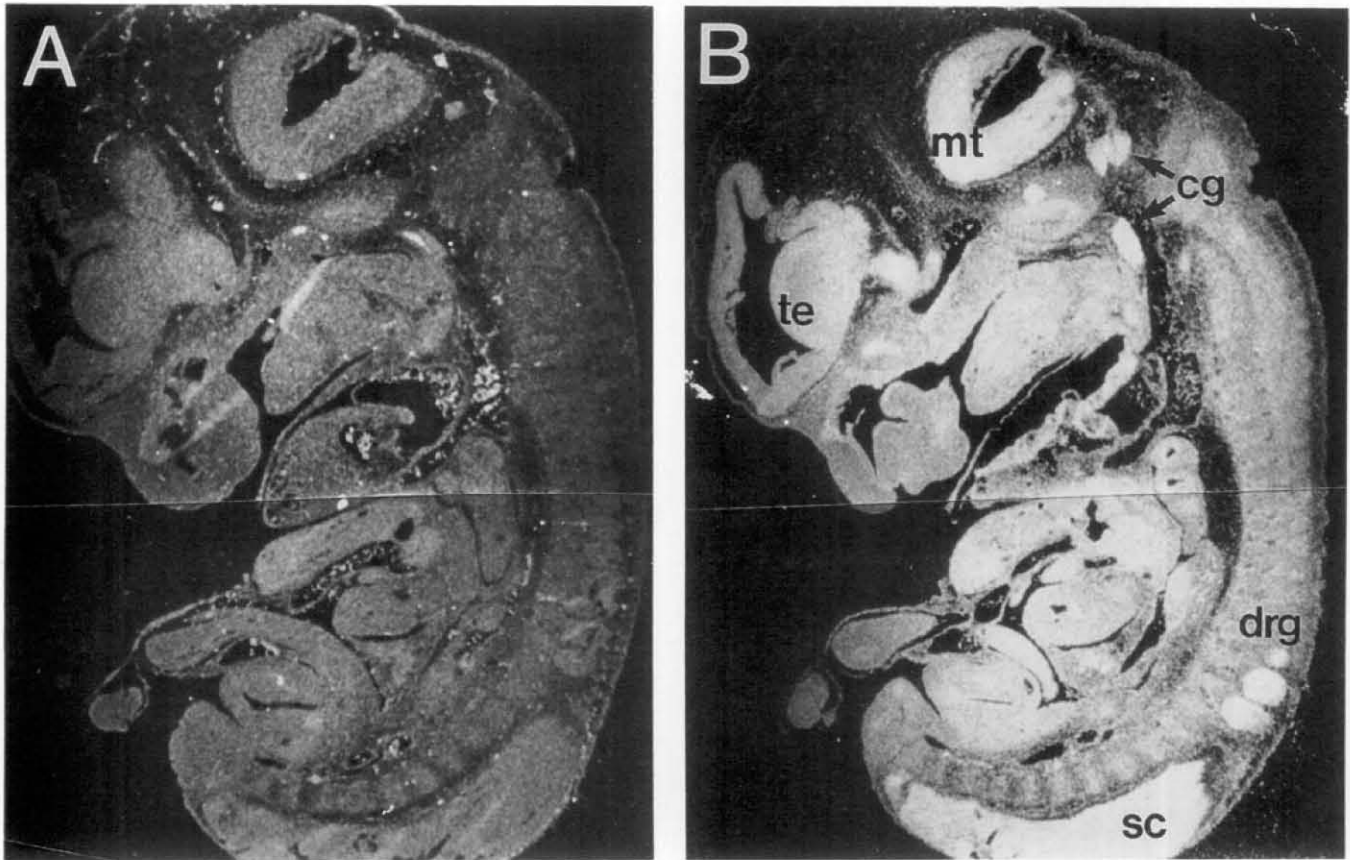
FIG. 7. *Dvl-1* expression at 12.5 days postcoitum. (A) Embryonic Day 12.5 parasaggital section hybridized to *Dvl-1* sense probe. Low level of background signal is evenly distributed across the embryo. (B) Adjacent section hybridized to *Dvl-1* antisense probe. Expression is seen at a high, uniform level throughout the spinal cord (sc) and brain: te, telencephalon; mt, metencephalon. Expression is also high in dorsal root ganglia (drg) and most or all cranial ganglia (cg), including the vestibulocochlear ganglion (top arrow) and the inferior ganglion of the vagus nerve (bottom arrow). Significant, broadly distributed expression is also seen in many other tissues, notably the craniofacial mesoderm and most trunk organ systems.

sion are seen scattered throughout many other regions of the brain, such as the cerebral cortex (Fig. 8E). These patterns may correspond to the locations of neuronal cell bodies. The quality of the *in situs* was confirmed with independent antisense probes, whereas sense probes resulted in low background hybridization (data not shown).

At all times the most striking expression occurs in the granular cell layer of the cerebellum, the same region which expresses *En*-2 (Davis and Joyner, 1988). Because this layer of cells arises after birth, we followed the course of *Dvl-1* expression in the cerebellum throughout postnatal development. *Dvl-1* is expressed in the external germinal layer (EGL) from at least birth (Fig. 8D), and as these cells migrate inward to form the granular cell layer (GCL) the distribution of hybridization signal

FIG. 6. *Dvl-1* expression in midgestation embryos. Saggital sections were hybridized as described under Materials and Methods and stained with methyl green to visualize morphology. In these dark-field photographs the hybridization signal appears as white grains. Sense controls are in the left column and adjacent sections hybridized with an antisense probe in the right. (A) Embryonic Day 8.5, midsaggital section of an embryo undergoing turning (sense probe). (B) Adjacent 8.5 day section hybridized with antisense probe shows a moderate level of expression in most tissues, including the neurectoderm (ne) and neural groove (ng) as well as the head mesenchyme (hm), somites (so), and presomitic mesoderm. Expression is also seen in some extraembryonic membranes (ex). (C) Parasaggital section of 9.5 day embryo sense probe. (D) Antisense probing of 9.5 day embryo shows high expression throughout the CNS: forebrain (fb), midbrain (mb), hindbrain (hb), and spinal cord (sc). Expression is also seen in most other tissues, as earlier. (E) Midsaggital section of embryo at 10.5 days—sense probe. (F) Adjacent section hybridized to *Dvl-1* antisense probe. Expression is seen at a high, uniform level throughout the spinal cord (sc) and brain: te, telencephalon; di, diencephalon; me, mesencephalon; mt, metencephalon; and my, myelencephalon. Expression is also apparent in most of the developing organ systems of the trunk as well as in other tissues and is essentially ubiquitous.

changes accordingly. At Day 6, when the EGL reaches its maximum thickness even as cells have already begun to form the GCL, *Dvl-1* is expressed in two layers (Fig. 8E). By Day 22, when the cells of the EGL have all migrated inward through the molecular layer (ML), *Dvl-1* expression is seen only interior to the ML (Fig. 8G). *Dvl-1* may also be expressed in the Purkinje cells, which occur as a monolayer between the GCL and the ML, but at lower levels (data not shown). In addition, we detected high levels of *Dvl-1* expression in the cerebral cortex (Fig. 8E) and hippocampal formation (Figs. 8E and 8F) during postnatal development.

The GCL is primarily composed of granule neurons but also has other cell types, such as glia and golgi neurons. High magnification of GCL signals suggests that *Dvl-1* is primarily in the large neuronal nuclei (data not shown). This is supported by the observation that *Dvl-1* expression in the cerebellum of *Weaver* homozygotes is largely absent (P.S., J.K., and R.N., unpublished observations). In *Weaver* mutants, granule cell neurons proliferate in the EGL but fail to migrate inward to form the GCL, and die in the most internal region of the EGL (Rezai and Yoon, 1972; Rakic and Sidman, 1973; Sotelo and Changeux, 1974; and Smeyne *et al.*, 1991). Thus, in the cerebellum, as in other areas of the brain, *Dvl-1* expression seems to be largely neuronal.

DISCUSSION

We have isolated a mouse homolog of the *Drosophila dsh* gene that shares 50% identity (65% similarity) at the amino acid level. The *Dvl-1* gene, which maps to distal mouse chromosome 4 (Beier *et al.*, 1992), encodes a 695-amino-acid protein with a calculated molecular weight of 75,312 Da. Hydropathy analysis suggests that both proteins have similar structures in regions showing little or no amino-acid conservation as well as in homologous domains. Analysis of the *Dvl-1* protein sequence did not reveal any probable function of this protein; however, it was found to contain a region of similarity to the "undefined domain" of *dlg*, a *Drosophila* tumor suppressor gene (Bryant and Woods, 1992; Klingensmith *et al.*, 1994). While the *dlg* encoded protein does not contain a transmembrane domain, it has been localized to the cell membrane, at the site of septate junctions. This "undefined domain" is also shared by the erythrocyte protein p55. p55 is a protein that, like *dlg*, contains an SH3 domain, a guanylate kinase domain, and is associated with the cytoplasmic face of the plasma membrane (Ruff *et al.*, 1991). As *Dvl-1* is potentially involved in *Wnt* signal transduction, which may involve cell–cell junctions (reviewed by Nusse and Varmus, 1992), it is possible that this "undefined domain" is involved in either protein localization or interaction with the cortical cytoskeleton.

Substantial levels of expression of *Dvl-1* were detected by Day 9.5 postcoitum (p.c.) by RNase protection, increasing over the next 2 days. RT–PCR indicated expression from the earliest time checked—7.5 days p.c., at the beginning of neurogenesis. We do not know when *Dvl-1* is first expressed in embryogenesis, but it is transcribed in embryonic stem cells (data not shown). *In situ* hybridization of 7.5 day embryos revealed expression of *Dvl-1* throughout the neural folds and in the mesoderm, with an area of exclusion around the node. Expression is stronger by Day 9.0, with *Dvl-1* transcripts throughout most of the embryo, with high levels in the CNS. At Day 12.5 abundant expression is detected in the dorsal root ganglia and many if not all cranial ganglia, as well as in all parts of the brain and spinal cord. Many other tissues also express *Dvl-1* during their early development, such as liver, lungs, and limb buds. *Dvl-1* is also expressed in at least some extraembryonic membranes.

The *Dvl-1* gene is expressed in a number of adult tissues. The highest levels of expression were detected in testis and brain and substantial amounts in kidney, breast, and muscle. A moderate level of *Dvl-1* was found in adult heart (data not shown). In the brain, expression of *Dvl-1*, from birth through adulthood, occurs in areas of high neuronal density, such as the hippocampus, olfactory bulb, and cerebellum. Within the cerebellum, expression is primarily in the granular cell layer, very similar to the pattern seen with *En-2* (Davis *et al.*, 1988).

Neither the deduced protein sequence nor the expression pattern of *Dvl-1* reveals the molecular function *Dvl-1* encodes, which is also true of the *Drosophila* homolog, *dsh*. However, much is known about the function of *dsh* from phenotypic and mosaic analysis. Like *Dvl-1*, *dsh* expression is widespread or ubiquitous during development (Klingensmith *et al.*, 1994; Theisen *et al.*, 1994). However, *dsh* is not a general requirement for cell differentiation or viability but rather is involved in a few discrete processes of pattern formation (Klingensmith and Perrimon, 1991). Phenotypic and clonal analyses in a wide variety of contexts suggest that *dsh* is required in any given cell for that cell to respond appropriately to specific external cues (Klingensmith *et al.*, 1994; Theisen *et al.*, 1994). In light of the analyses of *Drosophila dsh*, we find the widespread, high-level expression of *Dvl-1* in the mouse CNS particularly intriguing. *Dvl-1* is abundant in the cells of the midembryonic CNS, when this tissue is growing very rapidly. In postnatal development, *Dvl-1* is expressed at high levels in neuron-rich areas. For example, *Dvl-1* is very abundant in the cells of the external germinal layer of the cerebellum as they migrate inward to form the granular cell layer. *Dvl-1* remains at high levels as the axons of the nascent granule neurons begin the process of outgrowth, pathfinding, and synapsis. Similarly, *Dvl-1* is abundant in the cerebral cortex and hippocampus during postnatal de-

velopment. In all these cases neural cells must respond to various external cues to achieve their proper fate. Perhaps *Dvl-1* is involved in this process.

In *Drosophila*, one external signal for which cells require *dsh* to respond is that encoded by *wingless*, the homolog of *Wnt-1*. In the ventral epidermis of the embryo, *wg* modulates the expression of *engrailed* and *armadillo* in a paracrine fashion; *dsh* mediates both effects in the target cells (Noordermeer *et al.*, 1994; Siegfried *et al.*, 1994). *dsh* has also been shown to function in the response of target cells to *wg* signals in several other embryonic and imaginal patterning events (Klingensmith *et al.*, 1994; Couso *et al.*, 1994). *dsh* is expressed in all cells of the ventral epidermis of the embryo (Klingensmith *et al.*, 1994), although only a subset of them normally responds to *wg*. Ectopic production of *wg* suggests, however, that all cells in this tissue are capable of responding to *wg* (Noordermeer *et al.*, 1992). If this reflects a general mechanism of action of *Wnt* genes, it suggests that the molecular machinery for *Wnt* reception may be relatively widespread, with the *Wnt* signals highly localized within responsive tissues. We find it interesting that *Dvl-1* is expressed in all tissues known to be responsive to *Wnt-1* (breast, Nusse and Varmus, 1982; salivary gland, Tsukamoto *et al.*, 1988; and midbrain, McMahon and Bradley, 1990, Thomas and Cappechi, 1990) as well as in testis, in which *Wnt-1* is expressed, yet has no known function (Shackleford and Varmus, 1987). *Wnt-1* is also expressed in the hindbrain and spinal cord, yet *Wnt-1* knockouts have no phenotype in these tissues. An explanation for this is that other *Wnt* genes expressed in these tissues interact with the same signal transduction pathways (Roelink and Nusse, 1991; McMahon *et al.*, 1992). When *Wnt-1* is expressed ectopically throughout the dorsal–ventral axis of the spinal cord, it elicits a response in a broad distribution of cells, including those which would not normally ever encounter *Wnt-1* (Dickinson *et al.*, 1994). We find that *Dvl-1* is expressed throughout the hindbrain and spinal cord during this period, consistent with the hypothesis that *Dvl-1* plays a role in the signal transduction pathway mediated by multiple *Wnt* genes.

As mentioned above, in *Drosophila* embryos *dsh* is required by *engrailed*-expressing cells to respond to *wg*, revealing that in at least some contexts *dsh* is in the signal transduction pathway effecting *en* expression. The earliest known defect of *Wnt-1* mutants in mice is a loss of *En-1* expression at the 5-somite stage in the midbrain (McMahon *et al.*, 1992). *Dvl-1* is present in these tissues as well as *En-1*. Thus, it is possible that, as occurs during fly development, *dishevelled* and *engrailed* have a functional relationship in mammalian development. Consistent with this hypothesis are the overlapping expression patterns of *En-2* and *Dvl-1* observed throughout cerebellum development.

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