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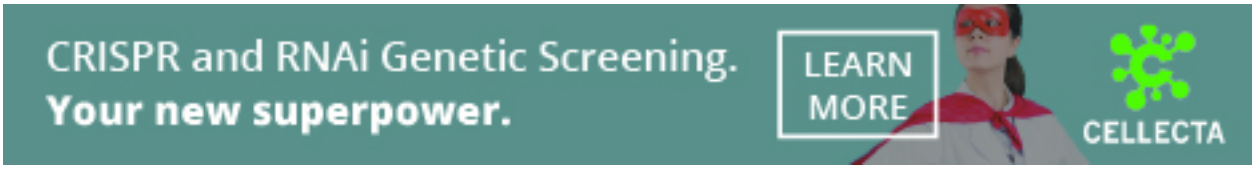

RESEARCH

Organization and Promoter Analysis of the Mouse *Dishevelled-1* Gene

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We have characterized the genomic organization of a mouse homolog (*Dvl-1*) of *Drosophila dishevelled*, a segment polarity gene required for *wingless* signal transduction. The *Dvl-1* gene is organized into 15 exons ranging in size from 68 to 1315 bp spanning a region of 12,409 bp, with the largest and smallest intron being 5545 and 71 bp, respectively. Sequence analysis of the 5′-flanking region of the gene revealed a high GC content, six CCGCCC Sp-1-binding motifs, CREB, LBP-1 (leader-binding protein 1), and TGGCA-binding consensus sites. However, neither TATA or CAAT boxes are present, a characteristic shared by other GC-rich promoters. The 5′-flanking region has strong promoter activity when placed upstream of the luciferase gene. Promoter–luciferase constructs have demonstrated that the promoter is functional in transfection assays and that its activity is orientation dependent. Promoter deletions were used to define the 5′ and 3′ boundaries for promoter activity and revealed the presence of both positive and negative regulatory elements. Multiple transcription initiation sites were mapped by primer extension analysis and confirmed by reporter gene assay.

In insects and vertebrates, intercellular signaling molecules of the *wingless/Wnt* (*wg/Wnt*) gene family control cell fate decisions (for review, see McMahon 1992; Peifer and Bejsovec 1992). In *Drosophila*, *dishevelled* (*dsh*) is required for *wg* signal transduction and regulation of pattern formation within individual segments (for review, see Klingensmith and Nusse 1994; Perrimon 1994). Genetic epistasis studies indicate that *dsh* functions downstream of the *wg* signal to affect the expression of the homeo box gene *engrailed* (*en*) and cuticle differentiation (Klingensmith et al. 1994; Noordermeer et al. 1994). *dsh* also mediates *wg*-directed post-transcriptional accumulation of *armadillo* protein in cells including and flanking the *wg* stripe (Riggleman et al. 1990). In contrast to the nonautonomous behavior of *wg* in mutant cell clones, *dsh* acts autonomously (Klingensmith et al. 1994; Siegfried et al. 1994), suggesting a role in reception or transduction of the *wg* signal.

Members of the *wg* signal transduction pathway have been conserved through evolution. A family of mouse *wg* homologs have been isolated. One of these, *Wnt-1*, is involved in central nervous system (CNS) development (McMahon and Bradley 1990; Thomas and Capecchi 1990) as well as mouse mammary tumorigenesis (Nusse and Varmus 1982; Nusse et al. 1984; Tsukamoto et al. 1988). Likewise, the mouse *en* homologs, *En-1* and *En-2*, are involved in CNS development (Joyner et al. 1985, 1991; Joyner and Martin 1987; Wurst et al. 1994). The mouse *En* genes are initially coordinately expressed with *Wnt-1* during the definition of spatial domains in the developing CNS and then diverge by mid-embryogenesis (Davis et al. 1988; McMahon et al. 1992). The possibility that the *wg–en* pathway is conserved in mouse development is supported by the finding that maintenance of *En* expression in the cerebellum requires *Wnt-1* function (McMahon et al. 1992).

Previously, we have cloned the complete cDNA sequence of mouse *Dvl-1*, characterized its expression pattern in the embryo and adults (Sussman et al. 1994), and mapped the gene to distal mouse chromosome 4 (Beier et al. 1992). In the embryo *Dvl-1* is expressed in most tissues, with uniformly high levels in the CNS (Sussman et al. 1994). The gene is expressed throughout the developing brain and spinal cord, including those tissues and stages in which the *Wnt-1* and *En* genes are expressed. In adult mice, expression of the *Dvl-1* gene was found to be widespread, with brain and testis showing the highest levels.

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In the adult cerebellum, *Dvl-1* expression is confined to the granular cell layer, similar to the pattern seen for *En-2*.

Here we report the genomic organization of the mouse *Dvl-1* gene. We have sequenced the entire gene including 536 bp of 5′-flanking sequence and have determined the exon–intron structure of the gene. We have also identified its minimal promoter sequence by transfection of reporter constructs into mammary epithelial and neuronal cell lines.

RESULTS

Intron/Exon Organization

The *Dvl-1* genomic sequence was compared to the cDNA sequence (Sussman et al. 1994) to determine intron/exon structure. The *Dvl-1* gene contains 15 exons ranging in size from 68 to 1315 bp and spans 12,409 bp, with the largest intron being 5545 bp long and the smallest 71 bp long. The first 287 bp of exon 1 is noncoding, followed by 145 bp of coding sequence. The rest of the coding region lies on exons 2 through the first 374 bp of exon 15. The remaining 941 bp of exon 15 encompasses the 3′ untranslated region (Fig. 1). All exons and their junctions were sequenced twice. All intron–exon junctions conform to established consensus sequences (Mount 1982). Table 1 shows the exon sizes and relative position in the reported cDNA (Sussman et al. 1994), the 5′ splice donor sequences, estimated intron size, and 3′ splice acceptor sequences. Figure 2 shows the complete *Dvl-1* sequence of exons and their junctions.

Sequence of 5′-flanking Region

Subcloning and sequence analysis of the 536 bp of the 5′-flanking region of the *Dvl-1* gene revealed a high GC content and contains six CCGCCC repeats (Sp1-binding motifs), CREB (Faisst and Meyer 1992). LBP-1 (leader-binding protein 1) and TGGCA-binding consensus sites (Fig. 3). The presence of these sequences suggests that this region of the *Dvl-1* gene represents the promoter. The absence of TATA or CAAT boxes in the *Dvl-1* 5′-flanking region is characteristic of GC-rich promoters.

Determination of Transcription Initiation Sites

The transcription initiation sites of the *Dvl-1* gene were determined by primer extension using brain and testis RNA, tissues in which the gene is highly expressed. The primer extension was performed using an oligonucleotide complementary to position 106–132 of the MBG5 *Dvl-1* cDNA (Sussman et al. 1994). Four distinct extension products were observed (Fig. 4A). To confirm this result, primer extension was performed on RNA isolated from C57MG cells transiently transfected with a *Dvl-1* promoter–reporter construct. As seen in Figure 4B, three extension products, corresponding to transcription initiation at nucleotides 288, 244, and 157, were identical to those observed using brain and testis RNA. A prominent extension product 215 bp in length was observed in brain and testis RNA that was not seen in transfectant RNA (indicated by asterisk in Fig. 4A).

The most abundant extension products had lengths of 288, 244, and 215 bases and are upstream of the *Bss*HII site (Figs. 3 and 4). A third extension product, at moderate levels, has a length of 157 bases and is downstream of the *Bss*HII site. These results suggest that the start site of *Dvl-1* gene transcription is heterogeneous, occurring 25, 83, 112, and 156 bp upstream from the start of the MBG5 cDNA. This result was supported independently by RNase protection assay (Fig. 4) and a functional assay described below.

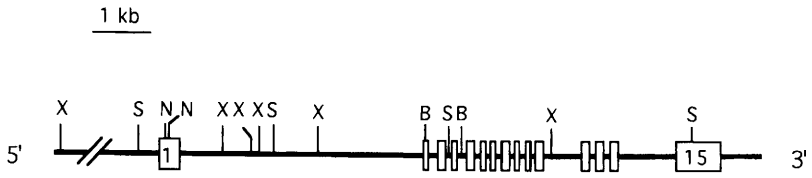

Figure 1 Physical map of the *Dvl-1* gene. The map was determined by restriction digests and sequencing of the λx6 clone. Exons are shown as open boxes. The restriction map of the region is presented for the following enzymes: *Bgl*I (B); *Not*I (N); *Sac*I (S); *Xba*I (X); (scale in kb is indicated to the *left*).

The 5′-flanking Region of the *Dvl-1* Gene Contains a Functional Promoter

To demonstrate that the 5′-flanking region of the *Dvl-1* gene contains a functional promoter, a series of chimeric *Dvl-1* promoter–luciferase gene constructs were pre-

Table 1. Exon and intron junctions of the mouse *Dvl-1* gene

Exon			Sequence at exon/intron junction		
no.	size(bp)	nucleotide position in cDNA	5′ splice donor	intron size (bp)	3′ splice acceptor
1	457	1–432	CTTCGGgtcagt	5545	ccacagGGTGGT
2	70	433–502	TCCTGGgtgagt	149	ttacagCTGGTC
3	122	503–624	CTTCAAgtaagg	224	atgcagACCAAA
4	104	625–728	ATGAGGgtacta	242	ccacagCTGCCC
5	139	729–867	CAGCCGgtgggt	97	cctcagGCTGAG
6	94	868–961	GACCGGgtaggc	71	ccgcagGCATCC
7	70	962–1031	ACATGGgtgagg	85	ctgcagAGAGGC
8	140	1032–1171	CTGCAGgtggga	78	ccgtagGTGAAC
9	77	1172–1248	GACAGGgtgagg	91	ctgtagGCCCAT
10	68	1249–1316	CAAGGGgtgagt	126	atgcagCTGACC
11	153	1317–1469	CCCCACgtaagt	510	ctgcagAGCTTG
12	132	1470–1601	TCATTGgtgagt	98	gcccagGGGCGG
13	168	1602–1769	GCAGTAgtgagt	85	tgccagACCTCG
14	207	1770–1976	GTGAAGgtgaga	1182	ccctagGGAGCA
15	1315	1977–3292			

pared (Fig. 5). The largest of these constructs, p4.8 *Dvl-1*–LUC cloned into the PXP1–LUC vector was assayed first and displayed promoter activity. From this plasmid a series of 5′ deletions were constructed and cotransfected into two cell lines, C57MG (mammary epithelial derived cell line) and C17-2 (neonatal cerebellar derived cell line), with an alkaline phosphatase reporter gene (pCMVSEAP). These cell lines were chosen because *Dvl-1* is expressed at a low levels in mouse breast tissue and at relatively high levels in the developing cerebellum (Sussman et al. 1994). The alkaline phosphatase activity resulting from the pCMVSEAP plasmid was used to standardize transfection efficiency. The relative promoter activities for the deletion constructs are shown in Figure 6. The 5′ boundary required for promoter activity (p0.6*Dvl-1*–LUC) mapped to the *Sac*I site located at position –536 relative to the 5′ end of the MBG5 cDNA (indicated as +1 in Fig. 3). The 5′ boundary is upstream of the major transcriptional start sites predicted by primer extension. Interestingly, the larger constructs p4.8*Dvl-1*–LUC and p2.0*Dvl-1*–LUC displayed relatively lower promoter activity, suggesting the possibility of upstream repressor-binding sites (Fig. 6A,C). In ad-dition, the promoter appears to be strictly unidirectional, as reversing the orientation of the insert p4.8 *Dvl-1*–LUC abolished promoter activity completely (Fig. 6A,C).

To ensure that we did not omit possible downstream transcription initiation sites, we constructed and assayed plasmid p0.7*Dvl-1*–LUC. The activity of the plasmid p0.7*Dvl-1*–Luc, which encompasses the sequence of p0.6*Dvl-1* and an additional 96 bases of the untranslated region of the cDNA up to the *Nae*I site, did not show a significant difference from the maximal promoter activity displayed by p0.6*Dvl-1*–LUC in the C17-2 cell line (Fig. 6D). Activity of p0.7*Dvl-1*–LUC was slightly lower than p0.6*Dvl-1*–LUC in the C57MG cell line (Fig. 6B). Thus, all major start sites appear to be upstream of the 5′ *Not*I site.

To confirm the sites of transcription initiation observed by primer extension we analyzed a series of 3′ deletions. A 3′ deletion from the *Not*I site to the *Bss*HII site (p0.5*Dvl-1*–Luc), a region where minor primer extension products were localized, decreased promoter activity by two- to three-fold (Fig. 6B,D). A 3′ deletion from the *Not*I site to the *Eco*47III site (p0.32*Dvl-1*–Luc) resulted in the abolition of promoter activity. Therefore, sequences between *Bss*HII and *Eco*47III are required for promoter activity. This region encompasses the consensus CREB (cAMP response element binding) site five of the CCGCCC repeats, and the initiation sites of the two most abundant extension products, located at positions 122 and 156 bp upstream from the start of the MBG5 cDNA.

DISCUSSION

In this paper we report the exon/intron mapping, sequencing, and promoter localization of the mouse *Dvl-1* gene. The *Dvl-1* gene contains 15

Figure 2 The nucleotide sequence of the mouse *Dvl-1* gene, displaying 262 bp of the 5′-untranslated region. Exon sequences are shown as boldface uppercase letters. The intron GT and AG dinucleotides are underlined. Intron sequences are displayed in lowercase letters. The start methionine (ATG) at position 263 and stop codon (TGA) at position 2720 are underlined. The entire sequence is present in GenBank under accession no. U28138.

exons within 12 kb of genomic sequence. The coding region begins in exon 1, 287 bp downstream of the 3′-most transcription initiation site. The rest of the coding region lies on exons 2 through the first 374 bp of exon 15. The remaining 941 bp of exon 15 encompasses the 3′-untranslated region.

Using primer extension we have mapped multiple transcription start sites upstream of the 5′ end of the *Dvl-1* MBG5 cDNA. The locations of the major start sites were confirmed using a functional reporter assay in cultured cells. These data indicate that transcription of RNA initiates at four major sites at positions –25, –83, –112, and –156 (Fig. 3) with respect to the 5′ end of MBG5 cDNA; however, we cannot rule out the possibility that some minor transcription sites do exist downstream of the *Bss*HII site (Figs. 3 and 4). The –83 initiation site was the only major primer extension product not detected in C57MG cells transfected with a reporter gene construct. It is possible that this is a consequence of having the promoter in multiple copies out of its usual chromosomal context or a true transcriptional difference because of the cell lines origin or maintenence in culture.

The minimal promoter sequence identified encompasses the sequences of the predicted major initiation sites, in that deleting the sequences in that region abolished promoter activity. In addition, this region encompasses the CREB consensus binding site, with CREB being most abundant in brain and binding to the cAMP response element (Faisst and Meyer 1992), and five of the CCGCCC repeats. We observed that constructs containing more extensive 5′-flanking DNA displayed lower promoter activity. This result suggests the presence of negative regulatory elements in the 1.4-kb sequence upstream of the construct p0.6*Dvl-1*–Luc.

Analysis of the 5′-flanking region of the *Dvl-1* gene shows that a 6-bp sequence, the GC box CCGCCC, is repeated six times in the promoter region at positions 90, 95, 130, 153, 178, and 484 bp upstream of the 5′ end of the MBG5 cDNA. Two GC box sequences, at positions –90 and –95, are found in an overlapping fashion (Fig. 3). This 6-bp GC box sequence is repeated six times within SV40 early promoter, located ~80 bp upstream from the RNA start site, and is essential for transcription (Benoist and Chambon 1981; Tijian 1981).

The features of the *Dvl-1* gene promoter region are very similar to those of a variety of proto-oncogene promoters, such as the human epidermal growth factor (EGF) receptor, the human Harvey *ras* (Ishii et al. 1985; Lu et al. 1994), and the human c-*src* promoters (Bonham and Fujita 1993), which contain repeats of the GC boxes with multiple transcription initiation sites, presumably because of lack of both TATA and CAAT box elements. Other genes with the same features have also been reported. It is interesting to note that all of these genes are involved in cellular growth control (Ishii et al. 1985). It is possible that the GC boxes in all these genes serve as attachment sites for DNA-binding pro-

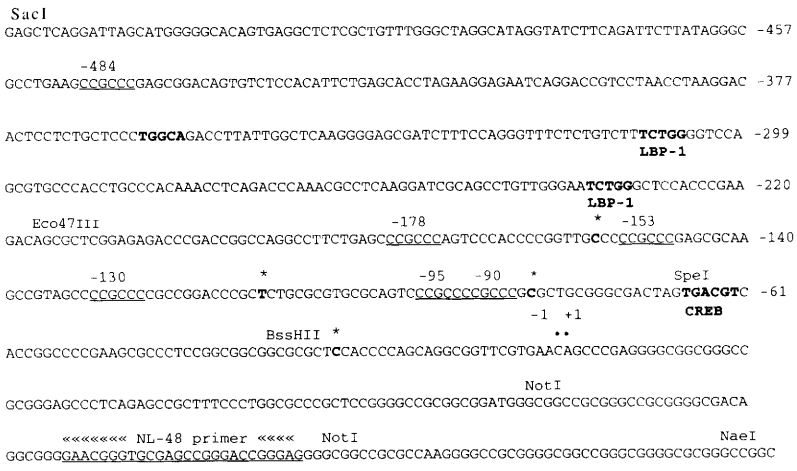

Figure 3 Sequence of *Dvl-1* 5′-flanking region. The sequence is numbered relative to the start of the MBG5 cDNA, which is marked as +1, and residues preceding it are represented by negative numbers. The major transcriptional initiation sites determined by primer extension and RNase protection are indicated by asterisks. The 26-mer oligonucleotide used as a primer for primer extension is underlined. Consensus sequences for factor-binding sites, identified using Quest software (Intelligenetics, Inc.), are indicated in boldface type. The repeated CCGCCC sequences are underlined.

teins. This has been shown in the human Harvey *ras* promoter in which Sp-1 acts as a start site selector (Lu et al. 1994). Gidoni et al. (1984) have also shown that the Sp-1 transcription factor, purified from HeLa cells, binds to the GC boxes of the SV40 promoter. Therefore, Sp1 or related factors may bind to *Dvl-1* promoter and play a role in its regulation.

Other potential transcription factor-binding sites in the 5′-flanking region of the *Dvl-1* gene include CREB and LBP-1, which is reported to recognize both consensus sequences 5′-TCTGG-3′ and 5′-CCAGA-3′, and TGGCA protein. LBP-1 has the interesting property of activating basal HIV-1 transcription from a downstream binding site (Jones et al. 1988). TGGCA protein is known to substitute for NF-I and acts as a transcription factor on viral and cellular enhancer/promoter elements (Ralph et al. 1987). The TGGCA-binding protein is also reported as a multifuctional DNA-binding protein, capable of serving a transcriptional role in the case of mouse mammary tumor virus, in addition to its known involvement in the replication of adenovirus (Miksick et al. 1987). It is possible that these sequences are needed for the regulation of *Dvl-1* transcription.

Determining the sequence and gene organization of the *Dvl-1* gene is an important step in performing detailed investigation of its regulation and for the elucidation of the role of *Dvl-1* in mammalian development.

METHODS

Mapping, Subcloning, and Sequencing of the Genomic Clone

Isolation of the mouse *Dvl-1* genomic clone is reported in Sussman et al. (1994). The genomic organization of *Dvl-1* was determined by restriction mapping, subcloning, and sequencing of the 21-kb genomic clone λx_6. This clone was mapped by partial and complete restriction digests using [γ-^{32}P]ATP end-labeled T3 and T7 oligonucleotides as probes for Southern analysis (Tartof and Hobbs 1988). The clone was mapped further using probes from the 5′ and 3′ ends of the *Dvl-1* cDNA clone MBG5 (Sussman et al. 1994; GenBank accession no. U10115). Based on the restriction map, genomic fragments were subcloned into the pBluescript KS vector (Stratagene) using established protocols (Maniatis et al. 1989). Templates for sequencing were generated by nested deletions using exonuclease III (Promega Erase-a-base kit). Subclones were sequenced by cycle sequencing with *Taq* polymerase using an Applied Biosystems 370A DNA sequencer. Ambiguous sequences were resolved by manual methods (U.S. Biochemical Sequenase v. 2, Cleveland, OH). Oligonucleotides were synthesized using a 391/392 DNA synthesizer (Applied Biosystems, Foster City, CA). Genomic sequences were compared to the *Dvl-1* cDNA sequence to determine intron/exon structure. Transcription factor-binding and promoter consensus elements were identified using the Intelligenetics Quest Software (Mountain View, CA).

Primer Extension Analysis

Primer extension analysis was done using the protocol of Ausubel et al. (1987). Antisense synthetic oligonucleotide 26 mers complementary to position 106–132 of the *Dvl-1* cDNA (Sussman et al. 1994; GenBank accession no. U10115) were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). Total RNA (20 µg) from indicated tissues (mouse brain and testis) and cell lines was mixed with 3×10^5 cpm of primer in a volume of 20 µl. The mixture was denatured at 75°C for 5 min, then 20 µl of 2× hybridization buffer (0.6 M KCl, 20 mM Tris-HCl at pH 7.6, 1 mM EDTA) and 0.5 µl of RNase inhibitor (Ambion) were added. The reaction mixture was overlaid with 100 µl of mineral oil and hybridized at 37°C overnight. After annealing, the mixture was extended with avian myeloblastosis virus (AMV) reverse transcriptase (Promega) for 40 min at 46°C. The extended products were phenol extracted, ethanol precipitated, heated at 65°C for 5 min, and analyzed by 8% denaturing polyacrylamide gel elec-

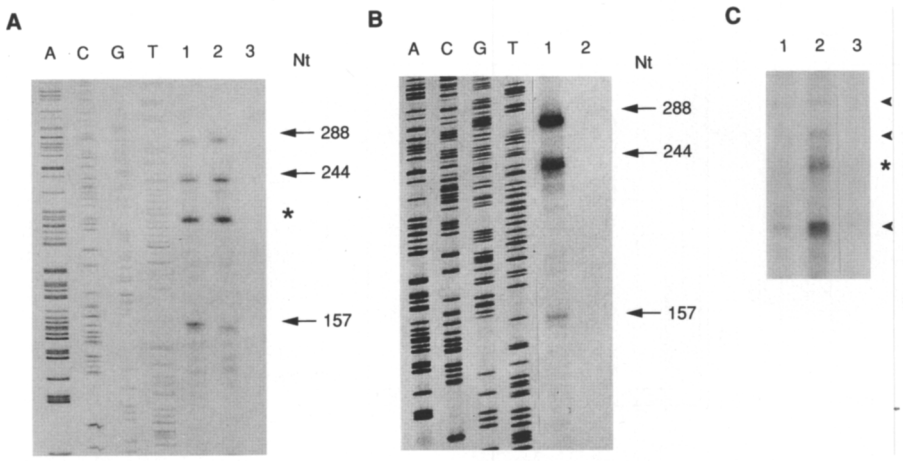

Figure 4 *Dvl-1* transcription start site mapping by primer extension and RNase protection analysis. An end-labeled oligonucleotide, complementary to position 106–132 of the *Dvl-1* cDNA, was used to prime reverse transcription of 20 µg of total RNA from brain (*A*, lane *1*), testis (*A*, lane *2*), tRNA (*A*, lane *3*; *B*, lane *2*), or reporter plasmid (p0.7*Dvl-1*–Luc)-transfected C57MG cells (*B*, lane *1*). Nucleotide sizes are indicated to the *right* of the M13mp18 sequencing ladder, which serves as a size marker. Arrows correspond to the major transcription initiation sites that match between the different RNA samples. The product marked by an asterisk (215 bp) is not detected in the transfected C57MG cell line. The autoradiograph was exposed for 24 hr. (*C*) Ribonuclease protection analysis (RPA) using a 415-nucleotide riboprobe generated from a genomic clone that spans the promoter and beginning of exon 1. Twenty micrograms of total RNA from brain (lane *1*), testes (lane *2*), and 10 µg of tRNA (lane *3*) was hybridized to the probe and processed as described in Methods. Protected products marked by arrowheads correspond in size to the transcription initiation sites that matched between primer extension analysis of tissues and transfected C57MG cells (288, 244, and 157 bp, respectively). The product marked by the asterisk corresponds to the initiation site only observed in primer extenstion of brain and testes RNA (215 bp). The tRNA serves as a negative control.

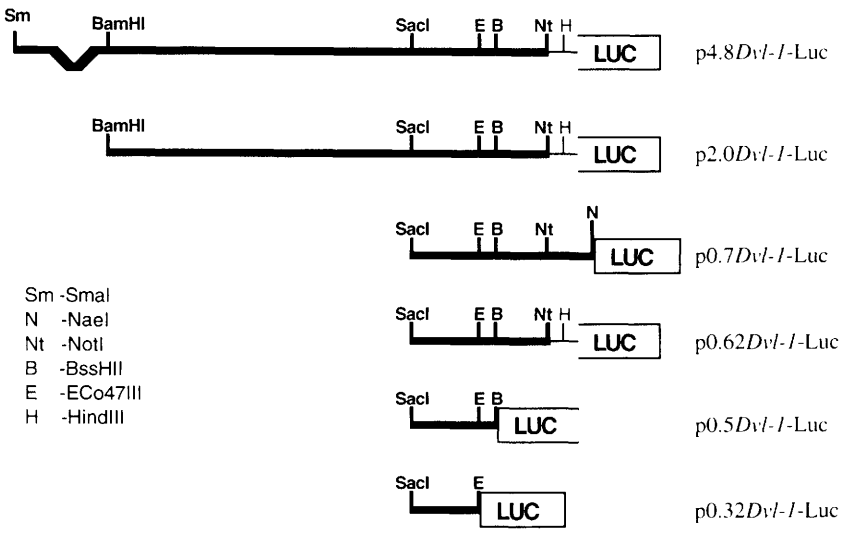

Figure 5 *Dvl-1*–Luc constructs. The names of each construct are shown at *right*, with the numbers preceding Luc referring to the size in kb of the 5′-flanking sequence. Restriction sites used in making the constructs are indicated. The *Dvl-1* regions are designated by bold lines.

trophoresis followed by autoradiography. An M13mp18 sequencing reaction was used as a size marker.

Ribonuclease Protection Analysis

A plasmid containing a 620-bp *Sac*I–*Not*I genomic fragment of *Dvl-1*, encompassing the 5′-untranslated region of exon 1 and upstream flanking region, was linearized using *Eco*47III, treated with 0.25 mg/ml of proteinase K, extracted using jthe Magic DNA clean up kit (Promega), and eluted in water. Antisense [α-^{32}P]UTP-labeled riboprobe (415 nucleotides in length) was generated using T3 RNA polymerase (MAXIscript in vitro transcription kit, Ambion Inc.).

Total cellular RNA was extracted from mouse brain and testes using the guanidinium isothiocyanate method (Chomczynski and Sacchi 1987). RNase protection analysis was performed as described by Melton et al. (1984). Total RNA (20 µg) was hybridized overnight at 50°C to 5×10^5 cpm of riboprobe in a total volume of 40 µl of buffer containing 50% formamide in PIPES buffer. Following hybridization, samples were digested for 30 min at room temperature in RNase digestion buffer (10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 0.3 M NaCl, 40 µg/ml of RNase A and 100 U/ml of RNase T1). Digestion was terminated by the addition of 20 µl of 10% SDS and 50 µg of proteinase K, followed by incubation for 15 min at 37°C. The samples were extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol in the presence of 20 µg of tRNA. Samples were resuspended in gel-loading buffer, heated at 95°C for 5 min, and resolved on a 6% polyacrylamide/8 M urea sequencing gel. A DNA sequencing reaction using M13mp18 template primed with –40 primer was used as a size standard.

Construction of luciferase *Dvl–1* plasmids

The Pxp1 luciferase vector (Nordeen 1988) was used to make the plasmid

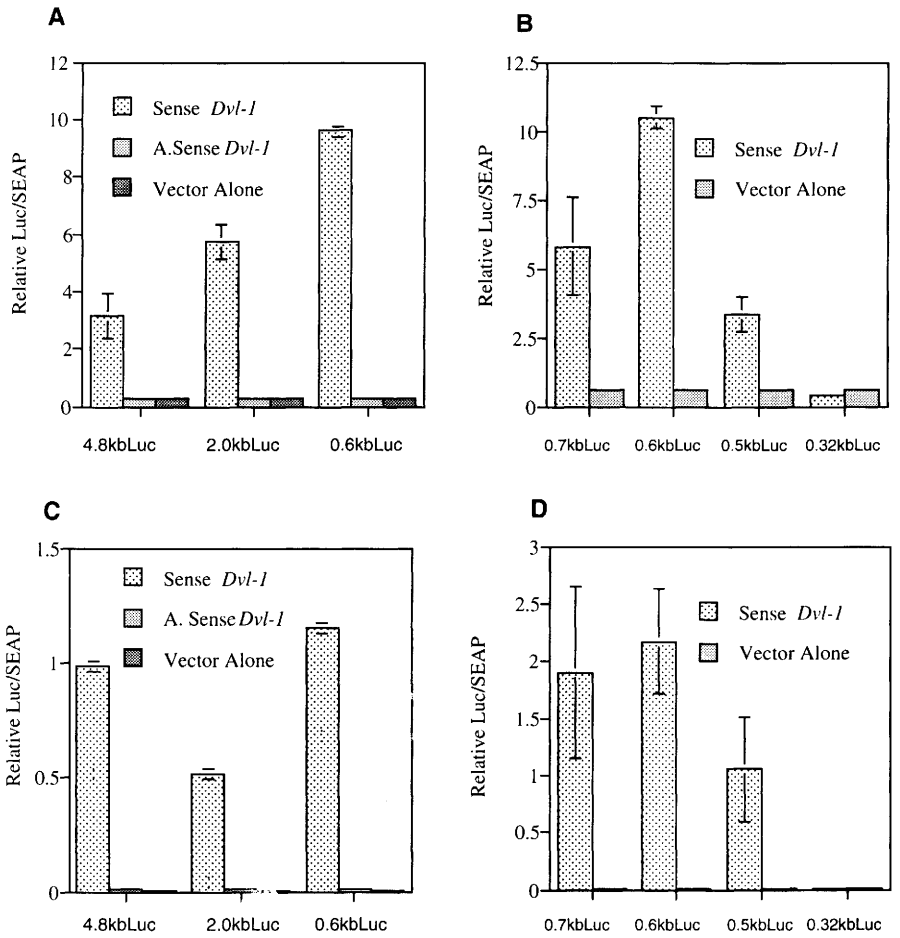

Figure 6 Promoter activity from transient transfection of 5′ *Dvl-1*–Luc constructs. (*A,B*) Results from a mammary epithelial cell line (C57MG); (*C,D*) a neuronal cell line (C17-2). For each construct 35-mm dishes were transfected in triplicate in the presence of pCMVSEAP, to normalize the transfection efficiency. SEAP and luciferase activities were assayed 48 hr after transfection. Promoter activity is expressed as a ratio of RLU per alkaline phosphatase activity (LUC/SEAP). Results are representative of three independent experiments.

p4.8*DVl-1*–Luc: A 5.6-kb *Xba*I fragment from the *Dvl-1* x_6 genomic clone, a region encompassing exon 1 and 4.8 kb of upstream sequence, was subcloned in the *Xba*I site of pKS (pKS–Xba5.6). pKS–Xba5.6 was digested with *Not*I, the 4.8-kb fragment was isolated and made blunt using Klenow polymerase, and subcloned in the pKS *Sma*I site (pKS–Sma4.8). The plasmid pKS–Sma4.8 was digested first with *Xba*I, the ends were blunted with Klenow, digested with *Hin*dIII (a site in the polylinker of pKS vector), and subcloned in the *Sma*I–*Hin*dIII site of the PXP1 luciferase vector (p4.8*DVl-1*–Luc).

A second plasmid, p2.0*DVl-1*–Luc, was constructed by cutting p4.8*DVl-1*–Luc with *Xho*I–*Bam*HI, to remove the 5′-most 2.8 kb of the *Dvl-1* promoter. The ends were filled in using Klenow, and self-ligated. Similarly, the third plasmid, p0.6*DVl-1*–Luc, which includes the first 109 untranslated nucleotides of the cDNA, was constructed by deleting part of the sequence of p4.8*DVl-1*–Luc construct with *Sac*I followed by self-ligation. The plasmid p0.7*Dvl-1*–Luc encompasses the sequence of p0.6*DVl-1* and an additional 96 bases of the untranslated region of the cDNA up to the *Nae*I site. The two smaller plasmids p0.5*Dvl-1*–Luc and p0.32*Dvl-1*–Luc were constructed by deleting part of the sequence of p0.6*DVl-1*–Luc construct, respectively, with *Bss*HII–*Hin*dIII and *Eco*47III–*Hin*dIII, followed by Klenow treatment and self-ligation (Fig. 5).

Transient Transfections of C57MG and C17-2 Cell Lines

The mouse C57MG mammary epithelial cell line (a gift from R. Nusse, Stanford University, CA) and the rat C17-2 neonatal cerebellum cell line (a gift from E. Snyder, Harvard University, Cambridge, MA), were grown at 37°C, 5% CO_2, in Dulbecco's modified Eagle medium (DMEM, GIBCO, Grand Island, NY) containing 10% fetal bovine serum (plus 5% horse serum for C17-2 cell lines), 2 mM glutamine, and antibiotics. Cells were seeded at 1×10^5/35-mm dish and grown to 70% confluency. The *Dvl-1* promoter–luciferase constructs were cotransfected with an alkaline phosphatase reporter construct (pCMVSEAP) using lipofectamine (GIBCO BRL). For each transfection, 1 μg of *Dvl-1*–LUC with 0.2 μg of pCMVSEAP and 8 μl of lipofectamine reagent were diluted separately in 100 μl of serum-free medium. The two solutions were combined and the mixture was incubated for 45 min at room temperature to allow DNA–liposome complexes to form. For each transfection, 0.8 ml of serum-free medium was added to the tube containing the complexes and overlaid on to the PBS-rinsed cells. At 9–10 hr post-transfection 1 ml of DMEM-20 [20% fetal bovine serum (FBS)] was added to each plate. After 24 hr the transfection reagent was removed, cells were fed with DMEM-10 (10% FBS), and incubated for another 24 hr before collecting conditioned medium and cell extracts. Transfections were performed in triplicate. The alkaline phosphatase activity resulting from the pCMVSEAP plasmid was used to normalize transfection efficiency. pCMVPXP1, a cytomegalovirus promoter–enhancer-driven luciferase plasmid, and PXP1 vector alone (Nordeen 1988) were used as positive and negative controls.

Reporter Assay for Secreted Alkaline Phosphatase

Chemiluminescent detection of secreted placental alkaline phosphatase (SEAP) was performed using the chemilumi-

nescent reporter assay kit Phosphalight (Tropix, Bedford, MA). Fifty microliters of 1× dilution buffer was mixed with 15 µl of conditioned media collected from 35-mm plates and incubated at 65°C for 30 min, to inactivate the endogenous phosphatase activity. Following a brief chill on ice, duplicate 25-µl aliquots were added to 25-µl aliquots of assay buffer in wells of a microtiter plate and incubated at room temperature for 5 min. Reaction buffer (25 µl) was then added to wells in 5-sec intervals. After a 20-min incubation at room temperature, plates were read using a monolight-1000 luminometer (ML1000 Dynatech, Chantilly, VA) with settings of 1 sec delay, 5 sec integrals. The activity per transfection is expressed as relative light units (RLU) for the average of duplicate readings (signal values were subtracted from the background values).

Reporter Assay for Luciferase

For luciferase assays, the cell extracts were prepared by Triton X-100 lysis (Ausubel et al. 1987). Cells were washed twice with ice-cold PBS and extracted in 100 µl of luciferase extraction buffer (1% Triton, 25 mM glycylglycine at pH 7.8, 15 mM EGTA, 1 mM DTT). Samples were spun at 12,000*g* for 5 min, and 45 µl of supernatant was added in duplicate to microtiter wells containing 165 µl of luciferase assay buffer (25 mM glycylglycine, 15 mM potassium phosphate at pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 1 mM DTT). Luciferase activity was measured as relative light units following injection of 90 µl of the substrate (luciferin) into a Dynatech monolight-1000 luminometer at 1 sec delay and 20 sec integrals. The activity per transfection is expressed as RLU for the average of duplicate readings (signal) with background values subtracted. Luciferase activity for each transfection was normalized by dividing Luc activity (RLU) by SEAP activity and reporting the mean and standard deviation for a set of triplicate transfection experiments.

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