Dorsoventral patterning in the *Drosophila* central nervous system: the *intermediate neuroblasts defective* homeobox gene specifies intermediate column identity

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One of the first steps in neurogenesis is the diversification of cells along the dorsoventral axis. In *Drosophila* the central nervous system develops from three longitudinal columns of cells: ventral cells that express the *vnd/nk2* homeobox gene, intermediate cells, and dorsal cells that express the *msh* homeobox gene. Here we describe a new *Drosophila* homeobox gene, *intermediate neuroblasts defective* (*ind*), which is expressed specifically in the intermediate column cells. *ind* is essential for intermediate column development: Null mutants have a transformation of intermediate to dorsal column neuroectoderm fate, and only 10% of the intermediate column neuroblasts develop. The establishment of dorsoventral column identity involves negative regulation: *Vnd* represses *ind* in the ventral column, whereas *ind* represses *msh* in the intermediate column. Vertebrate genes closely related to *vnd* (*Nkx2.1* and *Nkx2.2*), *ind* (*Gsh1* and *Gsh2*), and *msh* (*Msx1* and *Msx3*) are expressed in corresponding ventral, intermediate, and dorsal domains during vertebrate neurogenesis, raising the possibility that dorsoventral patterning within the central nervous system is evolutionarily conserved.

[*Key Words*: vnd; NK2; tinman; neuroectoderm; central nervous system]

The *Drosophila* central nervous system (CNS) develops from a bilateral neuroectoderm that lies to each side of a narrow strip of ventral midline cells. Single neuroectodermal cells delaminate from the surface epithelium and move into the interior of the embryo to form neural precursor cells called neuroblasts. The early neuroblasts form an orthogonal grid of four rows (1, 3, 5, and 7) along the anterior-posterior (AP) axis and three columns (ventral, intermediate, and dorsal) along the dorsoventral (DV) axis. Subsequently, each neuroblast expresses a characteristic combination of genes and contributes a stereotyped family of neurons and glia to the CNS. Thus the earliest steps in patterning the CNS are the formation and specification of neuroblasts.

Neuroblast formation is regulated by two phenotypically opposite classes of genes: Proneural genes promote neuroblast formation, whereas the neurogenic genes inhibit neuroblast formation. Proneural genes encode a family of basic helix–loop–helix transcription factors that are expressed in 4–6 cell clusters at specific positions within the neuroectoderm. Embryos lacking the proneural genes *achaete/scute* or *lethal of scute* have a reduced number of neuroblasts (for review, see Skeath and Carroll 1994). Conversely, neurogenic genes are expressed uniformly in the neuroectoderm, and embryos that lack any one neurogenic gene function, such as *Notch* or *Delta*, develop an excess number of neuroblasts (for review, see Campos-Ortega 1995).

The generation of neuronal diversity begins with the specification of unique neuroblast identities along both the AP and DV axes. The *wingless*, *hedgehog*, *gooseberry*, and *engrailed* genes are expressed in stripes of neuroectoderm that subdivide the AP axis. They are required for establishing AP row identity within the neu-

roectoderm and neuroblasts (Chu-LaGraff and Doe 1993; Zhang et al. 1994; Skeath et al. 1995; Bhat 1996; Matsuzaki and Saigo 1996; Bhat and Schedl 1997; McDonald and Doe 1997). For example, *gooseberry* is expressed in row 5 neuroectoderm. Embryos lacking *gooseberry* function have a transformation of row 5 into row 3 neuroectoderm and neuroblast identity, whereas misexpression of *gooseberry* results in the converse row 3 to row 5 transformation (Zhang et al. 1994; Skeath et al. 1995). Similarly, *wingless* encodes a protein secreted from row 5 and required for specifying the fate of the adjacent rows 4 and 6 neuroectoderm and neuroblasts (Chu-LaGraff and Doe 1993). Although we have learned a great deal about how the *Drosophila* CNS is patterned along the AP axis recently, relatively little is known about patterning along the DV axis.

Two genes are expressed in restricted domains along the DV axis within the neuroectoderm: *ventral nervous system defective* (*vnd*) is an NK2 class homeobox gene expressed in the ventral column neuroectoderm (Jimenez et al. 1995; Mellerick and Nirenberg 1995) and *muscle segment homeobox* (*msh*) is a homeobox gene expressed in the dorsal column neuroectoderm and neuroblasts (D'Alessio and Frasch 1996; Isshiki et al. 1997). Mutations in *vnd* cause defects in neuroblast formation and lead to severe defects later in neurogenesis (White et al. 1983; Skeath et al. 1994), but the role of *vnd* in patterning the neuroectoderm and neuroblasts along the DV axis has not been determined. Mutations in *msh* result in a partial transformation of dorsal neuroblasts into a more ventral or intermediate column identity, without affecting neuroblast formation (Isshiki et al. 1997). Signaling via the EGF receptor is required to establish ventral and/or intermediate column fates in the neuroectoderm (Rutledge et al. 1992; Raz and Shilo 1993; Schweitzer et al. 1995). Although *vnd* and *msh* are candidate genes for establishing ventral and dorsal column fates within the CNS, no genes are known currently to be expressed specifically in the intermediate column of the CNS.

In this paper we describe the identification and genetic characterization of a new homeobox gene, *intermediate neuroblasts defective* (*ind*) (GenBank accession no. AF095926), that is the first gene known to be expressed specifically in the intermediate column of neuroectoderm and neuroblasts. We show that *ind* function is required for the establishment of intermediate column identity in the neuroectoderm, and for the formation of intermediate column neuroblasts. In this paper and in McDonald et al. (1998), we examine the regulatory interactions between *vnd*, *ind*, and *msh*, and show that there is a hierarchical cascade of transcriptional repression. *Vnd* represses *ind* expression to establish the ventral boundary of *ind* transcription, and *ind* represses *msh* to establish the ventral boundary of *msh* transcription. The homeobox genes expressed in columns within the *Drosophila* neuroectoderm—*vnd*, *ind*, and *msh*—each have gene homologs expressed in corresponding domains along the DV axis of the vertebrate neural ectoderm. On this basis it appears that fundamental molecular mecha-

nisms of DV patterning may be similar in *Drosophila* and vertebrates.

Results

A screen for genes regulated by Tinman class homeodomain proteins

The *tinman* gene encodes a homeodomain protein required for heart and visceral mesoderm development (Azpiazu and Frasch 1993; Bodmer 1993). To discover how *tinman* directs mesodermal cell fates, we performed a screen to identify genes that are regulated by *tinman*. The screen relies on genetic selection in yeast for a protein–DNA interaction (Liu et al. 1993; Mastick et al. 1995). In brief, yeast expressing a gene encoding the Tinman homeodomain fused to the yeast GAL4 activation domain were transformed with a library of *Drosophila* genomic DNA fragments inserted upstream of a selectable marker in a reporter plasmid. When the fusion protein recognizes a *Drosophila* genomic DNA fragment it will activate transcription of the selectable marker. Genomic DNA fragments identified in the screen are therefore presumptive binding sites for the Tinman homeodomain.

Transcribed regions flanking the genomic DNA fragments isolated in the screen were identified and used as probes for whole-mount in situ hybridization to embryos. Several of the transcripts found in this way are produced in the mesoderm in patterns consistent with Tinman regulation, and will be described elsewhere (J.B. Weiss and M.P. Scott, in prep.). One of the identified genes, *ind*, located in polytene interval 71A, is transcribed in the CNS but not in the mesoderm. In this paper we describe the expression, regulation, and function of *ind* in patterning the DV axis of the CNS.

ind is a homeobox gene related to two homeobox genes expressed in the developing vertebrate CNS

The genomic DNA (Fig. 1A) adjacent to the putative Vnd binding site was employed as a hybridization probe to isolate full-length cDNA clones of *ind*. The predicted protein sequence shows that *ind* encodes a protein containing a homeodomain that is most closely related to the vertebrate Gsh1 and Gsh2 homeodomain proteins. There is 85% amino acid identity between Ind and either of the Gsh homeodomains (Fig. 1B). Ind, Gsh1, and Gsh2 also share a short amino-terminal region of homology (Fig. 1B). As is shown below, *ind* is expressed in two longitudinal stripes in sharply defined DV domains of the *Drosophila* CNS. This shows striking similarity to the expression of Gsh1 and Gsh2 in the developing murine CNS (Fig. 7 below, and Valerius et al. 1995; Szucsik et al. 1997).

ind is transiently expressed in the intermediate column neuroectoderm and neuroblasts

The temporal and spatial pattern of *ind* RNA accumula-

A

R0220

Vnd
binding site

ind
coding region

*ind*79.3

E   X   E   S   E   S   E X   X

━━ 1Kb

B

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Gsh2    1  MSRSFYVDSLIIKDSSRPAPSLPESHEGPDFFIPLGMPSPLVMSVSGPG.
Gsh1    1  MPRSFLVDSLVLREAS..DKKAPEGSPPPLF..PYAVPPPHALHGLSPGA
Ind     1  MSRSFLMDSLLSDRPNLSQKKEKLGSPVGSPTAAAAVAAAAMLPSIPMLP

Gsh2   50  CPSRKSGAFCV.CPLCVT.SHLHSSRPPAGAGGGATGTAGAAVAGGGVAG
Gsh1   47  CHARKAGLLCV.CPLCVTASQLHG..PP.....................
Ind    51  YPASVVGSYLFSLGIQQQQQQQQQQQQQQAAAAAAAAAAAAAALQQHPHVSS

Gsh2   98  GTGAL..PL..LKSQFSPAPGDAQF..C....PRVSHAHHHHHPPQHHHHH
Gsh1   72  GPPAL..PL..LKASFPPF..GSQY..C............HAPLGRQH.
Ind   101  SPGSLYHPYAQLFASKRKSSGFSNYEGCYPSPPLSANPNSQQLPIHHLY

Gsh2  139  HQPQQPG..SAAAAAAAAAAAAAAAALGH........PQHHAPVCAATTYN
Gsh1   99  .........SVSPGVAHGPAAAAAAAL...............YQTSYP
Ind   151  GSPVVGGLPLPEPGSFCTSPSASSSASLDYTNNFDEPQGKRFKHESSCSP

Gsh2  181  MSDPRRFHC....LSMGGSDTSQVPNGKRMRTAFTSTQLLELEREFSSNM
Gsh1  125  LPDPRRQFHC....ISV.DSSSNQLPSSKRMRTAFTSTQLLELEREFASNM
Ind   201  NSSPLKNESSGGPVEITPLINDYADSSKRIRTAFTSTQLLELEREFSHNM

Gsh2  227  YLSRLRRIEIATYLNLSEKQVKIWFQNRRVKHKKEGKGASRNNHT.....
Gsh1  170  YLSRLRRIEIATYLNLSEKQVKIWFQNRRVKHKKEGKGSNHRGGAGAGAG
Ind   251  YLSRLRRIEIANRLRLSEKQVKIWFQNRRVKQKKGGSESPTFN..LSTNS

Gsh2  271  .....SCKCVG.SQAHYARSEDEDSLSPASANEDKE...ISPL
Gsh1  220  GGAPQGCKCSSLSSAKCSEDDDELPMSPSSSGKDDRDLTVTP-
Ind   299  NGSPQASPVSPQVKVHELKVEA~~~~~~~~~~~~~~~~~~~~~
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C

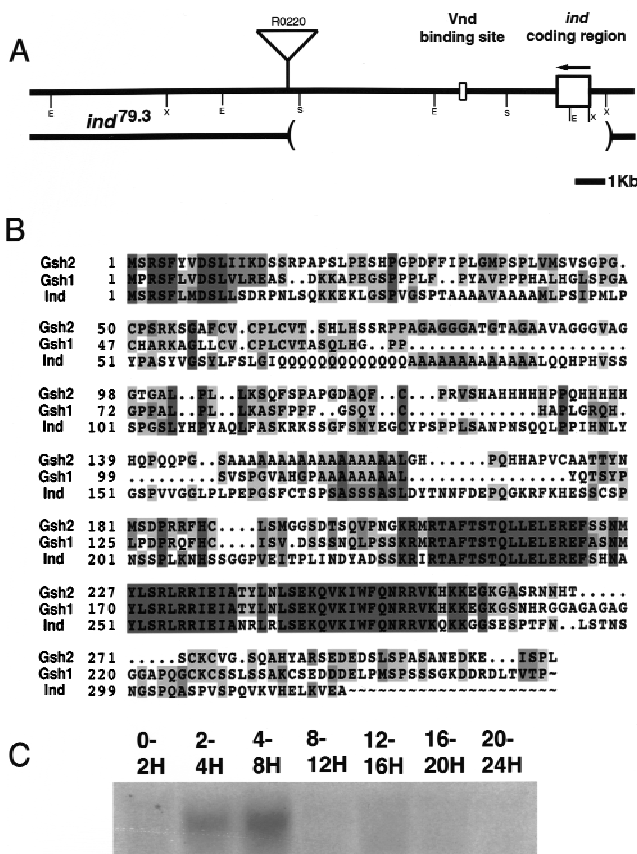|	0-2H	2-4H	4-8H	8-12H	12-16H	16-20H	20-24H

Figure 1. The structure of the *ind* genomic region, amino acid sequence of Ind, and developmental expression of *ind*. (*A*) The structure of the *ind* gene in wild-type and a mutant allele, *ind*79.3. (*Top*) The structure of the wild-type allele. The coding sequence for *ind* is contained in a single exon, shown as a large box. *ind* is transcribed in the direction indicated by the arrow. The binding site for Vnd, marked by a smaller box, lies 3 kb 3′ to the coding sequence. The insertion site of a P element, R0220, in relation to the gene is shown. Restriction endonuclease sites are marked with single letters: (E) *Eco*RI, (S) *Sal*I, (X) *Xho*I. (*Bottom*) The extent of a deletion that was generated by mobilization of the R0220 P element. The deletion is 7 kb long and removes the coding sequence for *ind*. (*B*) The predicted amino acid sequence of Ind aligned with two closely related mouse genes, *Gsh1* and *Gsh2*. The alignment shows conservation primarily within the homeodomain (amino acids 227–286 in Ind) though there is also a short region of homology at the amino terminus. Identities are marked in dark gray, similarities in light gray. Numbers at the left indicate the amino acid positions in the respective proteins. (*C*) An RNA blot of staged embryos hybridized with a radiolabeled *ind* cDNA clone. The times in hours postfertilization of the embryo collections are marked above the lanes. All lanes were loaded with 20 µg of total RNA.

tion was determined using RNA blots (Fig. 1C) and whole-mount in situ hybridization (Fig. 2). A single 1.3-kb *ind* transcript appears at 2–4 hr of development with peak accumulation at 4–8 hr of development; no transcripts were detected later in embryogenesis (Fig. 1C). Whole-mount in situ hybridization first detects *ind* expression in two parallel, lateral columns in stage 5 cel-

lular blastoderm embryos (Fig. 2). At this stage each column is about five cells wide and runs from the procephalic region to the most posterior region of the embryo (Fig. 2). During gastrulation, the *ind* expression domain narrows until each column is two cells wide. The position of each of the two symmetric *ind* columns is just dorsal to the domain of *vnd* transcription (Fig. 4, below), in cells that will become the intermediate column of neuroectoderm. When neuroblast formation begins at late stage 8, *ind* mRNA is expressed in both the intermediate column neuroectoderm and in the S1 neuroblasts (Nbs) derived from the intermediate column (Nbs 3-2 and 5-3). By stage 9, *ind* mRNA is absent from the neuroectoderm, but is detectable in all of the neuroblasts in the intermediate column (Nbs 3-2, 4-2, 5-3, 6-2, and 7-2). *ind* is not expressed in any ventral or dorsal column neuroectoderm or neuroblasts. By stage 11, *ind* mRNA is
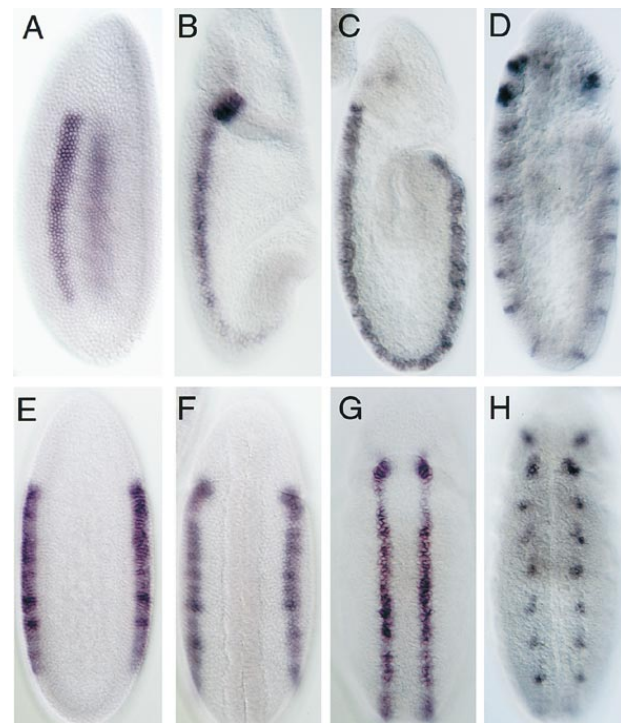
Figure 2. *ind* expression in wild-type embryos. Shown are lateral and ventral views of whole-mount in situ hybridizations with the *ind* cDNA to a developmental series of wild-type embryos. The embryos on the *top* (*A–D*) are shown in lateral views with anterior up and ventral to the left. The embryos on the *bottom* (*E–H*) are ventral views again with anterior up. The embryos are approximately stage matched and represent a developmental series starting at the left. (*A,E*) Stage 5, cellular blastoderm, embryos in which *ind* is expressed in two longitudinal stripes five cells wide in the intermediate neurectoderm. (*B,F*) Stage 7 embryos, following ventral furrow formation and ventral migration of the neurectoderm. *ind* expression in these embryos is narrowed to longitudinal stripes three cells wide. (*C,G*) Early stage 9 embryos in which *ind* is expressed in the intermediate neurectoderm and S1, intermediate column neuroblasts. (*D,H*) Late stage 9 to early stage 10 embryos. At this stage *ind* mRNA is progressively restricted to neuroblasts 6-2 and 7-2.

detectable in just a single intermediate column neuro-blast, Nb 6-2. After stage 11, *ind* mRNA was not detected anywhere in the embryo.

Monoclonal and polyclonal antibodies against the Ind protein were used to determine the subcellular localization of *ind* protein, assay the extent of *ind* posttranscriptional regulation, and confirm the identity of *ind*-expressing cells using double labels with other antibody markers. The Ind protein is detected in the nucleus at all stages assayed, as expected for a homeodomain protein (Fig. 3). We see no evidence for post-transcriptional regulation: *ind* mRNA and Ind protein patterns are virtually identical, except that the protein persists slightly longer than the mRNA (Figs. 2 and 3). The antibody staining confirms that Ind is restricted to the intermediate column neuroectoderm and neuroblasts. The Ind protein-containing cells are immediately adjacent but nonoverlapping with Vnd-containing cells of the ventral column neuroectoderm (see McDonald et al. 1998). Double labeling with various neuroblast markers shows that Ind is detected in the same intermediate column neuroblasts as *ind* mRNA (Figs. 2 and 3). In contrast to *ind* mRNA,

Ind protein is detectable in all intermediate column neuroblasts at stage 13, after the mRNA is no longer detectable in most of these neuroblasts. Ind protein is not detectable after stage 11 of embryogenesis.

ind is regulated by Vnd/Nk2 in the central nervous system

Although *ind* was identified in a screen for Tinman transcriptional targets, *ind* and Tinman are expressed in nonoverlapping, nonadjacent regions of the CNS and mesoderm, so it is unlikely that Tinman regulates *ind* directly. Furthermore, *tinman* mutant embryos have no change in *ind* expression (data not shown). We therefore tested the hypothesis that *ind* is transcriptionally regulated by Vnd, a homeodomain protein related closely to Tinman. Vnd is produced in the ventral neuroectoderm immediately adjacent to the *ind*-expression domain.

Genetic and molecular data demonstrate that *ind* is transcriptionally repressed by Vnd (Figs. 4, 5). In wild-type embryos the two genes are expressed in adjacent but nonoverlapping portions of the neuroectoderm. *vnd* is expressed in the ventral column, whereas *ind* is ex-
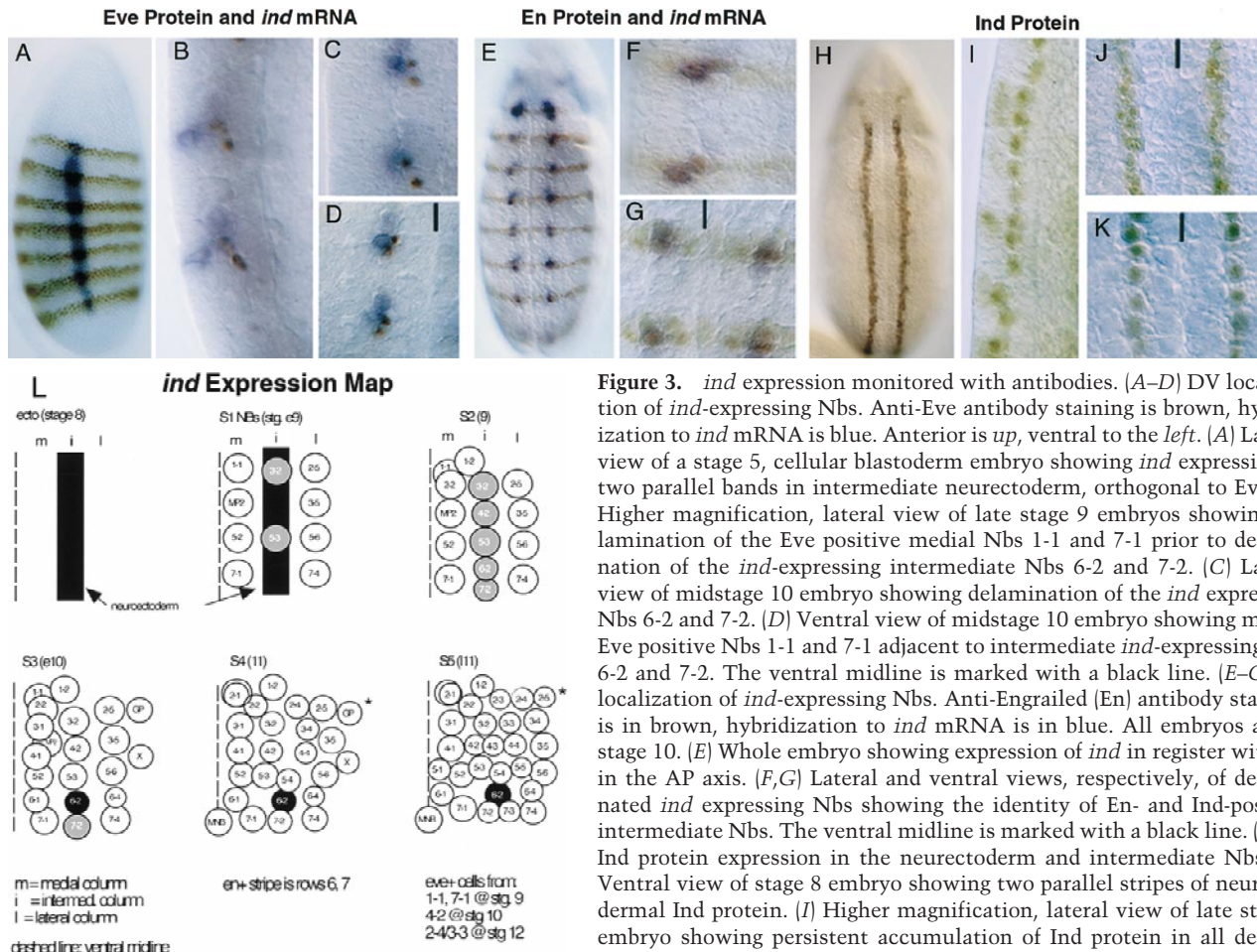

Figure 3. *ind* expression monitored with antibodies. (*A–D*) DV localization of *ind*-expressing Nbs. Anti-Eve antibody staining is brown, hybridization to *ind* mRNA is blue. Anterior is *up*, ventral to the *left*. (*A*) Lateral view of a stage 5, cellular blastoderm embryo showing *ind* expression in two parallel bands in intermediate neurectoderm, orthogonal to Eve. (*B*) Higher magnification, lateral view of late stage 9 embryos showing delamination of the Eve positive medial Nbs 1-1 and 7-1 prior to delamination of the *ind*-expressing intermediate Nbs 6-2 and 7-2. (*C*) Lateral view of midstage 10 embryo showing delamination of the *ind* expressing Nbs 6-2 and 7-2. (*D*) Ventral view of midstage 10 embryo showing medial Eve positive Nbs 1-1 and 7-1 adjacent to intermediate *ind*-expressing Nbs 6-2 and 7-2. The ventral midline is marked with a black line. (*E–G*) AP localization of *ind*-expressing Nbs. Anti-Engrailed (En) antibody staining is in brown, hybridization to *ind* mRNA is in blue. All embryos are at stage 10. (*E*) Whole embryo showing expression of *ind* in register with En in the AP axis. (*F,G*) Lateral and ventral views, respectively, of delaminated *ind* expressing Nbs showing the identity of En- and Ind-positive intermediate Nbs. The ventral midline is marked with a black line. (*H–K*) Ind protein expression in the neurectoderm and intermediate Nbs. (*H*) Ventral view of stage 8 embryo showing two parallel stripes of neurectodermal Ind protein. (*I*) Higher magnification, lateral view of late stage 9 embryo showing persistent accumulation of Ind protein in all delaminated intermediatecolumn NBs. (*J*) Higher magnification, ventral view of stage 8 embryo showing intermediate neurectoderm presence of Ind. (*K*) Higher magnification ventral view of stage 9 embryo showing Ind in all intermediate-column Nbs. The ventral midline is marked with a black line. (*L*) *ind* expression map diagram of neuroblast formation in one hemisegment stages 8–11. Ventral view, anterior is *up*, the midline is at the *left* of each panel. *ind* expression is shown in gray and black. Neuroblasts are designated by their final row number–column number.
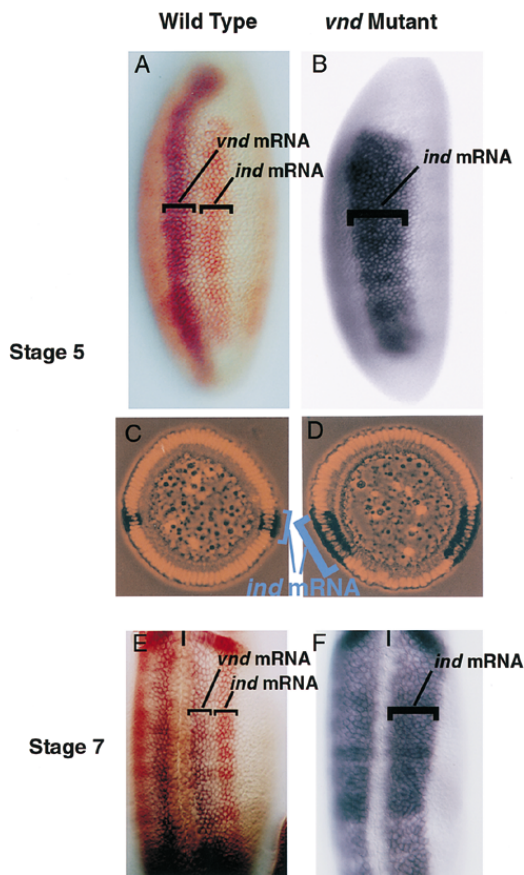
Wild Type   *vnd* Mutant

Figure 4. *ind* and *vnd* expression in wild-type embryos. Ventral derepression of *ind* in *vnd* mutant embryos. (*A–D*) Lateral (*A,B*) and cross sectional (*C,D*) views of wild-type (*A,C*) and *vnd* mutant (*B,D*) stage 5 embryos. Anterior is *up*, ventral to the *left* in *A* and *B*. In *C* and *D* ventral is *down*. (*A*) *ind* (red) is dorsal and adjacent to *vnd* (blue) in wild-type embryos. (*B*) In *vnd* mutant embryos *ind* (blue) expression is broadened ventrally to encompass the *vnd* expression domain. (*C,D*) Cross sectional views of wild-type (*C*) and *vnd* mutant (*D*) stage 5 embryos stained with *ind* (blue-black) and propidium iodide to mark nuclei. The ventral expansion of *ind* in *vnd* mutants is seen clearly in these cellular blastoderm embryos. (*E,F*) Ventral views of wild-type and *vnd* mutant stage 7 embryos. Anterior is *up*, the ventral midline is marked with a black line. (*E*) As above *ind* (red) is dorsal to *vnd* (blue). (*F*) In the *vnd* mutant *ind* (blue) expands ventrally to encompass the neurectoderm and the *vnd* expression domain.

pressed in the intermediate column. In *vnd* mutant embryos, *ind* expression is broader and encompasses what would normally be the *vnd*-expression domain. This can be observed clearly in lateral views of whole-mount embryos as well as in embryo cross sections (Fig. 4). These genetic experiments show that *vnd* is required to repress *ind* expression within the ventral column neuroectoderm.

To determine whether Vnd regulates *ind* transcription directly, we used bacterially expressed Vnd protein to perform electrophoretic mobility-shift and footprinting assays with the genomic *ind* DNA fragment identified in

our initial screen (GenBank accession no. AF096297). Vnd specifically binds the fragment of *ind* genomic DNA isolated in the screen (Fig. 5A). Three specific binding sites of roughly equal affinity can be identified using footprinting assays (Fig. 5B). The three sites protected in the footprinting assay each contain one copy of the sequence GTGAACT, which has been found to be a recognition sequence for both Vnd and the Tinman-related Nkx2.5 vertebrate protein (Chen and Schwartz 1995; Gruschus et al. 1997).
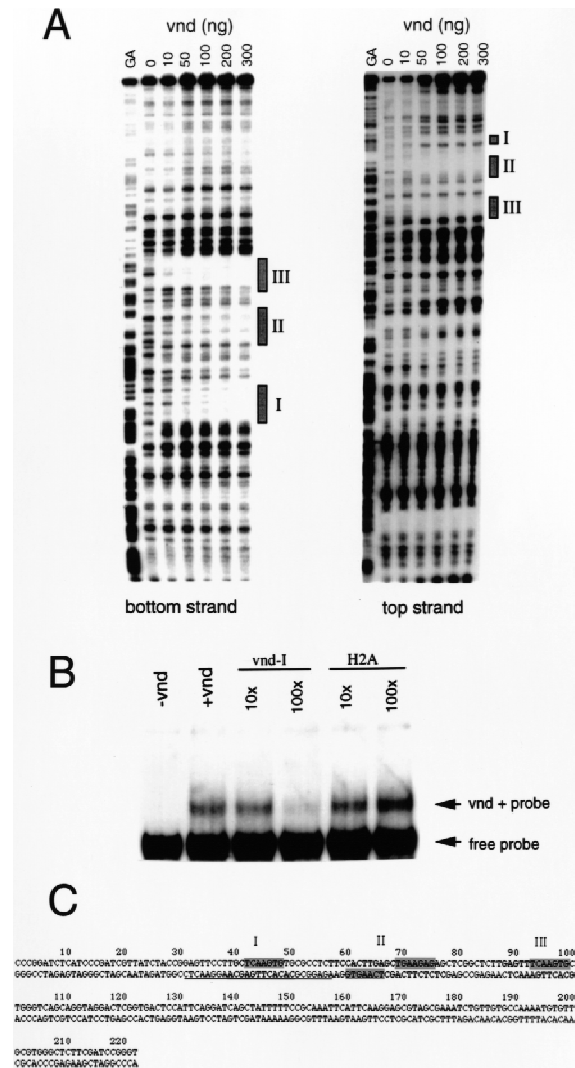

Figure 5. Binding of the Vnd protein to *ind* sequences. (*A*) DNase I footprints of both strands of the *ind* DNA. Three major protected regions become visible as the amount of recombinant Vnd homeodomain is increased. (*B*) Electrophoresis mobility-shift assay using *ind* DNA nucleotides 5–135, which includes all three Vnd-binding regions. A DNA/protein complex is observed upon addition of Vnd. This complex can be competed out with increasing amounts of the Vnd-I oligonucleotide but not with an irrelevant oligonucleotide (H2A). (*C*) Sequence showing the Vnd-binding regions as determined by DNase I footprinting. The protected regions are shaded in light gray, whereas a candidate consensus sequence is shaded in dark gray. The Vnd-I oligonucleotide sequence is underlined.

*ind is required for the specification
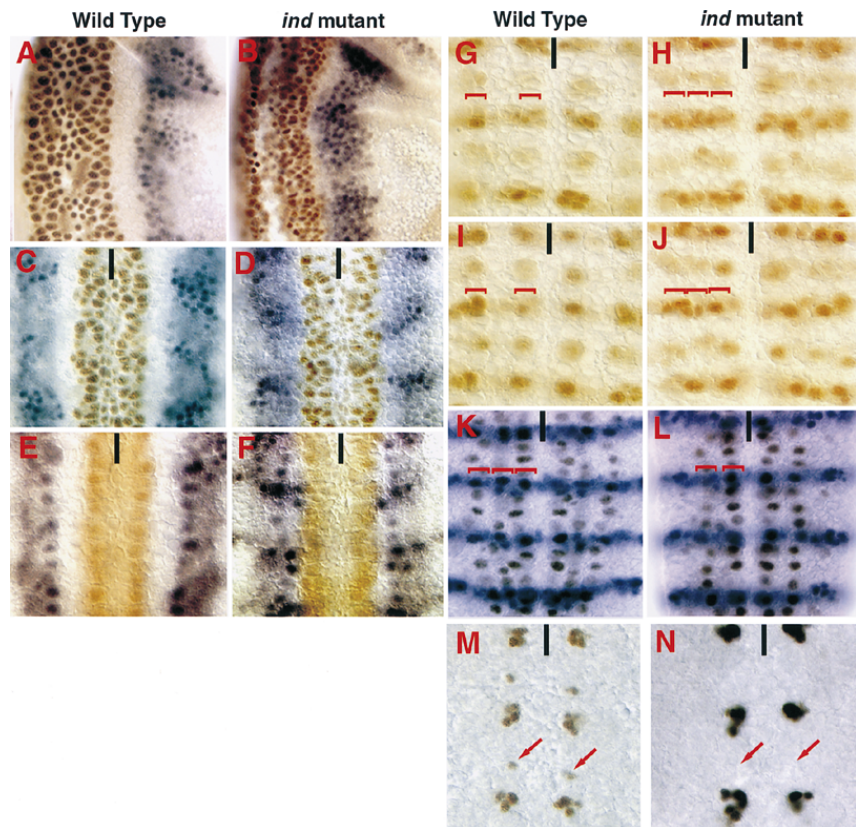of intermediate-column neuroectoderm
and neuroblasts*

ind null mutants were generated in two different screens. We identified a single *ind* allele (*ind*^{RR108}) in an ethyl methane sulfonate mutagenesis screen for altered *even-skipped* (*eve*) expression in the CNS (J. Skeath and C.Q. Doe, unpubl.). In addition, three *ind* alleles (*ind*$^{16.2}$, *ind*$^{79.3}$, and *ind*$^{4.1}$) were obtained by mobilizing a P element located next to the *ind* locus. All four alleles appear to be null mutations in *ind* for the following reasons: (1) one allele, *ind*$^{79.3}$, has a deletion of the *ind*-coding region (Fig. 1); (2) *ind*$^{16.2}$ and *ind*^{RR108} homozygous embryos have no detectable Ind protein during neurogenesis (data not shown); (3) *ind*$^{16.2}$ in *trans* with a large deficiency shows the same phenotype as the *ind*$^{16.2}$ homozygotes (data not shown); and (4) all four *ind* alleles have the same phenotype when homozygous (data not shown). We believe that the *ind* phenotype described below is caused specifically by the loss of *ind* function because the *ind*$^{79.3}$ mutation is a small deficiency that deletes the *ind*-coding region and 6 kb of flanking DNA, but not any other detectable gene (Fig. 1).

The earliest *ind* phenotype is observed in stage 7 embryonic neuroectoderm, when *msh* expression occurs both in its normal locations in the dorsal columns and in the adjacent intermediate columns (Fig. 6A,B). Thus *ind* represses transcription of *msh* directly or indirectly within intermediate column neuroectoderm. Normally the *ind* and *msh* expression domains are adjacent but nonoverlapping, consistent with negative regulation of *msh* by *ind*. During the earliest stage of neurogenesis (stage 8), wild-type embryos have expression of the proneural gene *achaete* in rows 3 and 7 of the neuroectoderm, with expression restricted to the ventral and dorsal columns and excluded from the intermediate column (Fig. 6G). *ind* expression in the intermediate column abuts these clusters of *achaete*-expressing cells precisely without overlapping them. In *ind* mutant embryos, derepression of *achaete* expression is observed within the intermediate column of neuroectoderm in rows 3 and 7 (Fig. 6H). This is consistent with a transformation of intermediate to dorsal neuroectoderm, as is seen at stage 7 with the *msh* marker. We conclude that *ind* represses *msh* and *achaete* gene expression directly or indirectly, and that *ind* is necessary for establishing proper intermediate-column identity within the neuroectoderm.

In addition to regulating gene expression in the neuroectoderm, *ind* has an essential role in neuroblast formation and specification. In wild-type embryos, Hunchback staining reveals three columns of neuroblasts (ven-

Figure 6. Loss of *ind* function results in loss of intermediate-column neuroblasts and intermediate-column-derived neurons. All views are ventral except *A* and *B* which are lateral. Anterior is *up*, ventral to the *left* in *A* and *B*. The ventral midline is marked with a black line. (*A–F*) Embryos doubly labeled for *msh* protein (blue) and Vnd protein (brown). (*A,C,E*) Wild-type embryos, blank space delineates the domain of Ind expression. (*B,D,F*). *ind*$^{16.2}$ mutant embryos show Msh is expanded in the neurectoderm at stage 7 (*B*). This persists until stage 9 when the neuroblasts delaminate (*D,F*). (*G–J*) Achaete (Ac) is repressed in the neurectoderm and neuroblasts by Ind. In wild-type embryos Ac is expressed in four clusters per hemisegment in the neurectoderm (*G*) and in four neuroblasts per hemisegment (*I*). In *ind* mutant embryos Ac expression is derepressed in the intermediate column (*H*). When a neuroblast forms at these positions it expresses Ac inappropriately (*J*). (*K,L*) Hunchback, En double labels showing loss of intermediate-column neuroblasts in *ind* mutant embryos. Pan neural expression of Hunchback in wild-type late stage 9 embryos shows there are three columns of neuroblasts (*K*). In *ind*$^{16.2}$ embryos only two columns of S1 neuroblasts are observed. Ind is therefore required for formation of intermediate-column neuroblasts (*L*). (*M,N*) Ind is required for neurons derived from intermediate-column neuroblasts. Even-skipped staining is shown in brown. The embryos are stage 13 and two types of Even-skipped positive cells are seen per hemisegment in this focal plane. The larger clusters are neurons derived from medial column neuroblasts. They are unaffected in *ind* mutants (*N*). The smaller clusters, highlighted by arrows, are RP2/RP2sib neurons that are derived from intermediate-column neuroblasts. These neurons are absent in the *ind* mutants (*N*).

tral, intermediate, and dorsal; Fig. 6K). In *ind* mutant embryos, only 10% of the intermediate-column neuroblasts develop, although adjacent ventral and dorsal column neuroblasts form normally (Fig. 6L). The 10% of neuroblasts that develop in the intermediate column always express *achaete*, which is never detected in wild-type intermediate column neuroblasts (Fig. 6I,J).

We stained the infrequent neuroblasts that form in the intermediate column of *ind* mutant embryos with anti-Msh and anti-Vnd antibodies to determine if there is a transformation to either dorsal or ventral cell fates in these mutants. A total of 80 hemisegments were scored. In a wild-type embryo 400 intermediate neuroblasts would be expected. In the *ind* mutant embryo 32 were observed. Eleven expressed Vnd, 12 expressed Msh, and 9 expressed neither marker. Thus, *ind* is essential for the formation of intermediate column neuroblasts, and for the repression of ventral- and dorsal-specific genes within these neuroblasts.

To test whether intermediate-column neuroblasts that form in *ind* mutant embryos have the potential to generate cell lineages characteristic of intermediate column neuroblasts, we assayed development of the Eve-containing RP2 neuron, which is a motor neuron derived from the intermediate-column neuroblast 4-2 (Doe 1992; Chu-LaGraff et al. 1995). The pattern of Eve is a sensitive indicator for normal cell fates within neuroblast cell lineages (Duffy et al. 1991; Chu-LaGraff and Doe 1993; Yang et al. 1993, 1997; Zhang et al. 1994; Chu-LaGraff et al. 1995). In wild-type embryos, an Eve-protein-containing RP2 is found in every hemisegment (100/100), whereas in *ind* mutant embryos the Eve-containing RP2 is never detected (0/96) (Fig. 6M,N). This defect is caused in part by a failure in NB 4-2 formation, but is also likely to be caused by defects in NB 4-2 specification or cell division in the 10% of the NB 4-2s that appear to form. These data suggest that *ind* regulates intermediate column neuroblast-cell lineages.

Discussion

The *Drosophila* CNS develops from ventral, intermediate, and dorsal columns of neuroectoderm and neuroblasts. Each column runs longitudinally along the embryo, and each gives rise to a specific set of neurons and glia (Bossing et al. 1996; Schmidt et al. 1997). The ventral neuroectoderm and neuroblasts express the *vnd/nk2* homeobox gene (Jimenez et al. 1995; Mellerick and Nirenberg 1995), whereas the dorsal neuroectoderm and neuroblasts express the *msh* homeobox gene (D'Alessio and Frasch 1996; Isshiki et al. 1997). *ind* appears to be the missing regulator, a gene that precisely marks and controls the development of the intermediate column of neuroectoderm and neuroblasts. The DV control genes *vnd*, *ind*, and *msh*—together with the AP-patterning genes—constitute a Cartesian cell-fate determination system for the developing CNS.

Identification and expression of *ind*

tinman encodes a homeodomain protein that is required

for heart and visceral mesoderm development, and is transcribed only in the tissues it affects. *ind* is not expressed in the mesoderm, yet was identified in a yeast one-hybrid screen for transcriptional targets of Tinman. This paradox was resolved with the cloning of *vnd* (Jimenez et al. 1995) and the recognition that Vnd and Tinman proteins contain closely related homeodomains. Both genetic and molecular data demonstrate that *ind* is repressed by Vnd protein. DNA-binding assays show that Vnd protein binds to a sequence adjacent to the *ind* transcript unit; this interaction is likely to be relevant biologically because Vnd is necessary to repress *ind* transcription in the ventral column of neuroectoderm and neuroblasts. Other transcription units identified in the yeast Tinman target gene screen have expression patterns consistent with regulation by Tinman, and not Vnd, and will be described elsewhere.

ind is required for repression of dorsal-column neuroectodermal markers within the intermediate column

Embryos lacking *ind* function have profound defects in the development of the intermediate column of the CNS. The defects include a derepression of dorsal column markers within the intermediate-column neuroectoderm. The *msh* gene is expressed normally in the dorsal column of the neuroectoderm, but *ind* mutant embryos show ectopic *msh* transcription in the intermediate column. Similarly, *achaete* is expressed normally in the ventral and dorsal regions of the neuroectoderm, but *ind* mutant embryos show ectopic *achaete* in the intermediate column of neuroectoderm. Whether *ind* acts directly or indirectly to repress *msh* and *achaete* expression is unknown. Taken together, the *msh* and *achaete* data suggest that one function of *ind* is to prevent transcription of dorsal column genes within the intermediate column of the neuroectoderm.

ind promotes intermediate-column neuroblast formation

In stage 9 wild-type embryos, five neuroblasts constitute the intermediate column in each hemisegment. In *ind* mutant embryos, at most one intermediate-column neuroblast is observed in each hemisegment, whereas the normal number of ventral and dorsal column neuroblasts form. Why do *ind* mutants have reduced neuroblast formation? One possibility is that *ind* activates proneural gene expression in the intermediate column of neuroblasts. The only proneural gene known to be expressed in this domain is *lethal of scute*, which we have not assayed. However, the ectopic expression of the proneural gene *achaete* that we observe in the intermediate-column neuroectoderm should promote—not reduce—neuroblast formation. Alternative explanations for the failure to generate intermediate column neuroblasts in *ind* mutant embryos include the following. (1) Proneural clusters of the dorsal column expand to include cells of

the intermediate column, but still produce a single dorsal column neuroblast per row. This is consistent with the *achaete* expression in the intermediate-column neuroectoderm. (2) Intermediate-column neuroectoderm assumes a novel cell fate that is incompatible with neuroblast formation. This hypothesis is supported by data showing that alterations in neuroectoderm cell fate along the AP axis can lead to reduced neuroblast formation without affecting proneural gene expression (Chu-LaGraff and Doe 1993).

Our results suggest that *ind* may act in parallel to the known proneural genes to promote neuroblast formation in the intermediate column. Similarly, *vnd* is thought to promote neuroblast formation by proneural-dependent and proneural-independent pathways (Jimenez et. al. 1995; McDonald et al. 1998). Vnd and Ind could promote neuroblast formation by transcriptionally activating known or novel proneural genes, by transcriptionally repressing neurogenic genes (e.g., *Notch*), or by regulating genes currently unlinked to the proneural or neurogenic pathways.

Regulation of ind *expression along the DV axis of the CNS*

The three longitudinal columns of cells along the DV axis are defined by the ventral expression and function of *vnd*; the intermediate expression and function of *ind*; and the dorsal expression and function of *msh*. In this paper and in McDonald et al. (1998) we show that *vnd* function is required to establish the ventral border of *ind*: In the absence of *vnd*, expression of *ind* is observed in the ventral-column neuroectoderm and neuroblasts. How the dorsal border of *ind* expression is established is unknown. The absence of *msh* function from the dorsal column has no effect on *ind* expression. The activating regulators for *ind* expression are also unknown, although candidates include the Dorsal protein, which regulates DV patterning of the cellular blastoderm (for review, see Rusch and Levine 1996), and the epidermal growth factor (EGF)-receptor pathway, which regulates DV cell fate in the neuroectoderm (for review, see Schweitzer and Shilo 1997).

Parallels with vertebrate neural patterning

The vertebrate CNS develops from a dorsal neuroepithelium that is similar in many respects to the *Drosophila* ventral neuroectoderm. For example, in *Xenopus* the neural primordium is established by Chordin-mediated repression of BMP4 activity, whereas in *Drosophila*, the Chordin homolog Sog inhibits the BMP4 homolog Dpp to establish the neuroectoderm (for review, see Ferguson 1996; Bier 1997). In addition vertebrate orthologs of *vnd* and *msh* have been identified: *Nkx2.1* and *Nkx2.2* and *Msx1* and *Msx3*, respectively (Holland 1991; Barth and Wilson 1995). Like *vnd* expression, *Nkx2.2* expression is restricted to ventral regions of the nerve cord in the regions that give rise to motor neurons (Fig. 7). Similarly

msh expression in the dorsal neuroectoderm is mirrored by *Msx1* transcription in the dorsal neural tube (D'Alessio and Frasch 1996; Isshiki et al. 1997). The similar spatial distribution of *Nkx2.2* and *Msx1* and *Msx3* expression to that of *vnd* and *msh*, respectively, suggests a conserved mechanism of DV patterning across species (D'Alessio and Frasch 1996; Wang et al. 1996). Consistent with this, a putative *ind* ortholog, *Gsh1*, is expressed in symmetric lateral domains in the mouse neural tube (Fig. 7; Valerius et al. 1995). In Figure 7 the DV order of the *Drosophila* and mouse genes are clearly the same. It is also apparent that there are gaps between the expression domains of the three vertebrate genes that we have compared. We speculate that other related vertebrate genes will fill in these gaps. If the overall parallel is indicative of an evolutionary relationship, it will be of great importance to investigate what target genes might be regulated in common by the fly and vertebrate genes. Common targets might be revealing about ancient cell type diversification during neurogenesis.

Materials and methods

Yeast screen for genomic DNA bound by Tinman

The screen was performed according to the protocol of Liu et al. (1993). Amino acids 221–416 of the Tinman protein were cloned into the yeast vector pBM2463, the activator plasmid. This con-
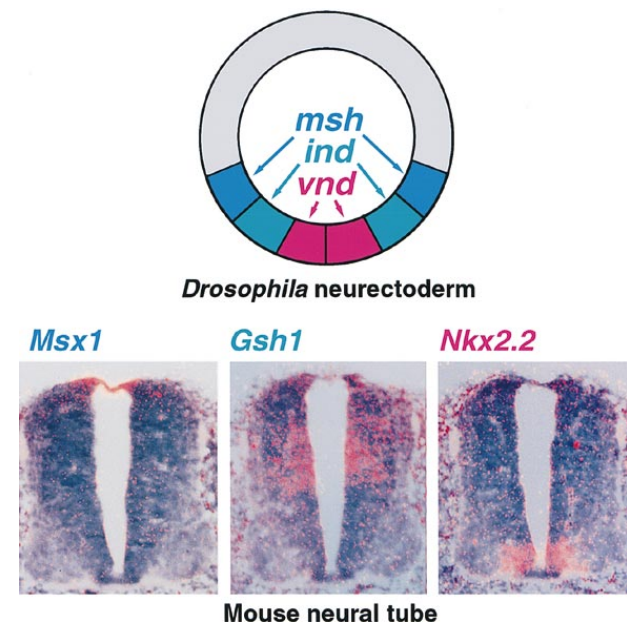

Figure 7. DV domains of expression of *Drosophila* and related mouse homeobox genes in the developing CNS. On the *left* a schematic cross section of a stage 7 *Drosophila* embryo showing the expression of *msh* (blue), *ind* (green), and *vnd* (red) in symmetric columns of neurectoderm. On the *right*, three panels show in situ hybridization with three related mouse genes performed on serial cross sections of the neural tube at the midthoracic level of an 11.5 day mouse embryo. Dorsal is *up*, ventral is *down*.

struct was transformed into yeast strain YM4271 by standard methods (Ausubel et al. 1994). The transformed yeast strain was subsequently transformed with a library of *Drosophila melanogaster* (DM) genomic DNA fragments cloned into the yeast vector pHR307a, the reporter plasmid (Mastick et al. 1995). The library construction is described in Mastick et al. (1995) and the library was provided by Javier Lopez. The library represents ~15% of the genome. Transformants (5×10^6) were screened for growth on medium lacking histidine. Secondary screening was performed for maintenance of the *Ade5* gene contained in the activator plasmid by a sectoring assay. Nonsectoring colonies were patched to medium lacking histidine and tested by replica plating for growth on medium lacking histidine in the presence of 5-fluoro-orotic acid (5-FOA). Growth on medium lacking histidine in the presence of 5-FOA acid implies that the activator plasmid coding for the Tinman fusion protein is not necessary for histidine auxotrophy. Positives were scored as yeast colonies that were nonsectoring on medium lacking histidine and unable to grow on medium lacking histidine in the presence of 5-FOA. Twenty-one independent positives were isolated from the screen. The reporter plasmids containing the Tinman binding sites were isolated from the yeast and the genomic DNA inserts subcloned and sequenced (Ausubel et al. 1994). Sequence analysis demonstrated that the 21 isolates represented 7 different genomic sites. Hybridization of these fragments to DM genomic DNA and polytene chromosomes confirmed that they are single-copy sequences of DM DNA (Pardue 1994).

Gene discovery

The genomic DNA inserts from the screen were employed as probes in hybridization screening for λ phage genomic clones by standard protocols (Sambrook et al. 1989). The library screened was provided by John Tamkun (Tamkun et al. 1992). Transcribed regions within the genomic λ were detected by hybridization to radiolabeled cDNA essentially as in Frei et al. (1986). Restriction digests of DM genomic λ phage that contained the Tinman binding sites were size fractionated in 0.8% agarose gel and transferred to a nylon filter (Sambrook et al. 1989). The filters were hybridized with radiolabeled cDNA synthesized by random priming of poly(A)-enriched RNA extracted from 0- to 24-hr embryos. Total embryonic RNA was extracted with guanidine HCl and organic solvents as described in Sambrook et al. (1989). Total RNA was enriched for poly(A)+ RNA by selection on oligo-dT cellulose according to the manufacturer's directions (Pharmacia, mRNA Purification Kit). Radioactive cDNA was synthesized according to the protocol in Sambrook et al. (1989) with the modification that SuperScript Reverse Transcriptase and Buffers were used (GIBCO-BRL). Hybridization of radioactive cDNA to genomic phage DNA was performed in Church buffer (Church and Gilbert 1984). Fragments of genomic DNA that hybridized to radiolabeled cDNA were employed as probes for whole-mount in situ hybridization to embryos and for hybridization screening of cDNA libraries. Full-length cDNA clones were isolated by standard hybridization screening from the staged embryonic cDNA library of N. Brown (Brown and Kafatos 1988). The sequence of both strands was determined by the cycle sequencing reaction and an automated ABI sequencer (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer). Homology to known genes was determined by BLAST search of the NCBI database (Altschul et al. 1997). The cytogenetic location of the gene was determined by hybridization to squashes of salivary polytene chromosomes by standard methods (Pardue 1994).

In situ hybridization

Whole-mount in situ hybridization to *Drosophila* embryos with both DNA and RNA digoxigenin-labeled probes was performed according to the protocol of Lehmann and Tautz (1994). For RNA probes proteinase K digestion was omitted. For double-label in situ hybridization both probes were RNA, one was labeled with fluorescein and the other was labeled with digoxigenin (Boehringer Mannheim). Following hybridization with both probes the embryos were incubated with alkaline-phosphatase-conjugated anti-fluorescein antibody. The first color reaction was developed with Fast-Red alkaline-phosphatase substrate (Boehringer Mannheim). After washing, the embryos were incubated in 0.1 M glycine HCl (pH 2.2), 0.1% Tween, for 10 min to remove the anti-fluorescein antibody (S. Small, pers. comm.). The embryos were washed again and the anti-digoxigenin antibody was added and the usual protocol for whole-mount in situ hybridization followed. For double-label experiments with whole-mount in situ hybridization and antibody staining the in situ hybridization was performed first without proteinase K digestion. Following development of the alkaline-phosphatase reaction antibody staining was performed as described below. For sectioning embryos were postfixed in 2% formaldehyde in PBS. Then 10-μm cryostat sections were made following embedding in gelatin according to Mastick et al. (1997). Briefly, following sequential infiltration in 5% sucrose and then 15% sucrose in 0.1 M phosphate buffer (pH 7.4), embryos were embedded in 7.5% gelatin, 15% sucrose in 0.1 M phosphate buffer. Cryosections were counterstained with propidium iodide and photographed.

In situ hybridization with radiolabeled probes to mouse embryos was performed according to the protocol of Angerer and Angerer (1992).

Isolation of mutants

A P-element insertion obtained from the *Drosophila* Genome Project was mapped near the coding sequence of *ind* (Fig. 1). This element was mobilized by a dysgenic cross and progeny tested for noncomplementation of a deficiency that uncovers *ind*. Six hundred lines from the dysgenic cross were tested for noncomplementation; 10 lines failed to complement the deficiency. Of these one was found to lack Ind protein by antibody staining (*ind*[16.2]). Additional complementation testing placed two other mutant lines in the same complementation group (*ind*[79.3] and *ind*[4.2]). All three showed the same CNS phenotype when homozygous or in combination with the deficiency. The *ind*[RR108] allele was identified in a large scale EMS mutagenesis of the third chromosome by assaying for changes in the number or pattern of Eve-protein-positive neurons (J. Skeath and C.Q. Doe, unpubl.).

The extent of the deficiency in *ind*[79.3] was determined by Southern analysis (Sambrook et al. 1989). The ends of the deletion were mapped by probes at both ends that recognize a common polymorphic fragment that is not recognized by fragments in the intervening interval.

DNase I footprinting and gel-shift analysis

The amino acid residues 563–723 containing the homeodomain of Vnd were inserted into the vector pRSET (InVitrogen) to generate a poly-histidine-tagged bacterial fusion construct. After induction, the bacteria were lysed in 6 M guanidine-HCl and the Vnd fusion protein was purified by nickel affinity chromatography. The protein was eluted in 8 M urea/sodium phosphate (pH 4.0). The purified Vnd fusion protein was dialyzed stepwise into 4, 2, and 1 M urea and finally into 0.5× Z-buffer [10% glyc-

erol, 12.5 mM HEPES (pH 7.8), 6 mM MgCl$_2$, 0.5 mM DTT, 50 mM KCl, 0.05% NP-40]. The final concentration of the Vnd fusion protein stock was 500 µg/ml.

DNase I footprinting was done essentially as described by Ryan et al. (1995). The *ind* repressor region was labeled using Klenow polymerase fragment to fill in the *Not*I or *Hind*III sites in the polylinker, for the bottom and top strands, respectively. The GA tract was generated by formic acid modification and piperidine cleavage (Maxam and Gilbert 1980). Approximately 20,000 dpm of DNA was added to increasing amounts of Vnd fusion protein in 0.5× Z-buffer with 1 µg of poly[d(I-C)]. After incubation for 15 min at room temperature, 0.2 unit of DNase I (Boehringer) was added and incubation proceeded for another 2 min. The reactions were stopped by adding 0.1 ml of STOP mix [50 mM Tris (pH 8.0), 100 mM NaCl, 1% SDS, 10 mM EDTA, 5 µg salmon sperm DNA, 50 µg protease-K] and the samples were digested overnight at 50°C. After phenol/chloroform extraction and ethanol precipitation, the samples were electrophoresed on 6% denaturing polyacrylamide gels.

Electrophoresis mobility-shift experiments used a 130-bp *Sau*3A fragment corresponding to nucleotides 5–135 of the *ind* repressor region and labeled by the Klenow fill-in reaction. Approximately 20,000 dpm of probe was incubated with 1 ng of Vnd fusion protein and a molar excess of specific and nonspecific competitor oligonucleotides. The double-stranded oligonucleotide corresponding to Vnd-binding site I, nucleotides 31–57, was used as a specific competitor, whereas the paired-domain binding site H2A (Adams et al. 1992) was used as a nonspecific control oligonucleotide. The binding reactions contained 0.5× Z-buffer and 1 mg of poly[d(I-C)]. After 15 min at room temperature, binding reactions were loaded onto 6% neutral acrylamide gels and run in 0.5× TBE.

Antibody generation and immunocytochemistry

Rat serum and monoclonal antibodies were raised against an Ind peptide conjugated to bovine serum albumin (BSA). After the monoclonal fusion, hybridoma supernatants were screened by ELISA against a peptide-KLH conjugate. Positive hybridoma lines were tested by immunohistochemistry. Embryo fixation, antibody staining, and identification of homozygous mutant embryos were performed essentially as by McDonald and Doe (1997). Embryos were staged according to Campos-Ortega and Hartenstein (1997). Primary antibodies were used at the following dilutions: mouse anti-Achaete monoclonal (990E5F1; 1:10; Skeath and Carroll 1992), rabbit anti-β galactosidase serum (1:3000; Cappel), rat anti-β-galactosidase monoclonal (1:10; Srinivasan et al. 1998), mouse anti-Eve [2B8; 1:10; (Patel 1994)], rabbit anti-Msh serum (1:500; T. Isshiki and A. Nose, unpubl.), rat anti-Ind serum (1:500), rat anti-IndN1 monoclonal (1:1), and rabbit anti-Vnd serum (1:10). Secondary antibodies of the appropriate species conjugated to biotin (Vector Labs) or alkaline phosphatase (Southern Biotechnology Associates) were used at a dilution of 1:400. Histochemical detection of primary antibodies was done with the HRP Vectastain Elite kit (Vector Labs) in conjunction with the Renaissance TSA Indirect kit (NEN Life Sciences) for some antibodies. After staining, embryos were dissected, mounted in 70% or 85% glycerol and examined on a Zeiss Axioplan microscope. Images were captured with a Sony DKC-5000 digital camera.

Acknowledgments

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