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Establishing neuroblast-specific gene expression in the *Drosophila* CNS: *huckebein* is activated by Wingless and Hedgehog and repressed by Engrailed and Gooseberry

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SUMMARY

The *Drosophila* ventral neuroectoderm produces a stereotyped array of central nervous system precursors, called neuroblasts. Each neuroblast has a unique identity based on its position, pattern of gene expression and cell lineage. To understand how neuronal diversity is generated, we need to learn how neuroblast-specific gene expression is established, and how these genes control cell fate within neuroblast lineages. Here we address the first question: how is neuroblast-specific gene expression established? We focus on the *huckebein* gene, because it is expressed in a subset of neuroblasts and is required for aspects of neuronal and glial determination. We show that Huckebein is a nuclear protein first detected in small clusters of neuroectodermal cells and then in a subset of neuroblasts. The secreted Wingless and Hedgehog proteins activate *huckebein* expression in distinct but overlapping clusters of neuroectodermal cells and neuroblasts, whereas the nuclear Engrailed and Gooseberry proteins repress *huckebein* expression in specific regions of neuroectoderm or neuroblasts. Integration of these activation and repression inputs is required to establish the precise neuroectodermal pattern of *huckebein*, which is subsequently required for the development of specific neuroblast cell lineages.

Key words: *huckebein*, *hedgehog*, *wingless*, *gooseberry*, *engrailed*, neuroblast, *Drosophila*

INTRODUCTION

Development of the *Drosophila* central nervous system (CNS) begins after gastrulation between embryonic stages 9-11, as single cells within the ventral neuroectoderm enlarge and delaminate into the embryo to form a stereotyped array of neural precursor cells, called neuroblasts (NBs; Campos-Ortega and Hartenstein, 1985; Doe, 1992). Neuroblasts divide asymmetrically to 'bud off' a series of ganglion mother cells (GMCs), which each produce a pair of neurons and/or glia. The type of neurons and glia generated is tightly correlated with the position of the neuroblast (Chu-LaGraff et al., 1995; Broadus et al., 1995; Bossing et al., 1996a,b); for example, the most anterior-medial neuroblast, NB 1-1, always produces the aCC/pCC neurons (Broadus et al., 1995; Bossing et al., 1996a,b). It has become clear that cell diversity in the CNS is generated in two steps: first, 'positional cues' in the neuroectoderm trigger neuroblast-specific gene expression (Chu-LaGraff and Doe, 1993; Zhang et al., 1994; Skeath et al., 1995; Skeath and Doe, 1996; Parras et al., 1996); and second, neuroblast-specific gene expression is required for the normal development of neurons and glia in the lineage (Doe et al., 1988a,b; Duffy et al., 1991; Cui and Doe, 1992; Mellerick et al., 1992; Higashijima et al., 1996; Chu-LaGraff et al., 1995; Bossing et al., 1996a; Lundell et al., 1996).

To understand fully how neuronal diversity is generated in the *Drosophila* CNS, we need to know how neuroectoderm position translates into neuroblast-specific gene expression, and how gene expression in a neuroblast leads to its characteristic cell lineage. In this paper we address the first question: how positional cues in the neuroectoderm control neuroblast-specific gene expression. We focus on the *huckebein* (*hkb*) gene for two reasons: first, it is expressed in a highly reproducible subset of neuroectoderm and neuroblasts (permitting easy identification of mutations altering the pattern); and second, it is required for GMC-specific gene expression and the specification of identified neurons and glia in at least four neuroblast lineages (Chu-LaGraff et al., 1995; Bossing et al., 1996a; Lundell et al., 1996). *hkb* is required within the NBs 1-1, 2-2, and 4-2 lineages for proper axon pathfinding of interneurons and motoneurons and for proper muscle target recognition by motoneurons (Chu-LaGraff et al., 1995; Bossing et al., 1996a). In addition, *hkb* is required for expression of *reversed polarity* (*repo*) in the A and B glia derived from the NB 1-1 lineage (Bossing et al., 1996a), for expression of *even-skipped* (*eve*) in the RP2 neuron derived from the NB 4-2 lineage (Chu-LaGraff et al., 1995), and is required for the production of serotonin in neurons derived from the NB 7-3 lineage (Lundell et al., 1996). Thus, *hkb* is an excellent candidate for investigating how positional cues in the neuroectoderm are transduced into lineage-specific cell fates in the *Drosophila* CNS.

hkb encodes a predicted zinc finger protein that is initially expressed in the blastoderm termini and endodermal anlage and is required for development of these structures (Weigel et al., 1990; Brönner et al., 1994; Reuter and Leptin, 1994). Within the CNS, *hkb* is expressed in a stereotyped pattern of neuroectodermal clusters and neuroblasts. The neuroectoderm can be divided into 7 rows (1-7, from anterior to posterior) and 3 columns (medial, intermediate and lateral), based on boundaries of gene expression and the arrangement of neuroblasts (Campos-Ortega and Hartenstein, 1985; Doe, 1992; Broadus et al., 1995). *hkb* is expressed in neuroectoderm of medial rows 1/2 and intermediate row 4, and subsequently in portions of rows 5 and 7; it is also transiently expressed in the neuroblasts that form at these positions (Chu-LaGraff et al., 1995).

What controls *hkb* expression in a subset of neuroectoderm and neuroblasts? The segment polarity genes are good candidates, because many are regionally expressed in the neuroectoderm at the time of *hkb* expression (reviewed by Perrimon, 1994), and many are required for normal CNS development (Patel et al., 1989a). Here we examine the function of four segment polarity genes – *engrailed* (*en*), *hedgehog* (*hh*), *wingless* (*wg*), and *gooseberry* (*gsb*) – in regulating *hkb* expression in the CNS.

en and *hh* are both expressed in neuroectoderm of rows 6/7, the posterior domain of each segment (Poole et al., 1985; DiNardo et al., 1985; Mohler and Vani, 1992; Tabata et al., 1992; Lee et al., 1992; Broadus et al., 1995). *en* encodes a nuclear homeodomain protein (Fjose et al., 1985); *hh* encodes a secreted protein that can be detected anteriorly in row 5 and posteriorly in rows 1/2 (Taylor et al., 1993; Tabata and Kornberg, 1994). In the embryo, *en* positively regulates *hh* expression in rows 6/7, and *hh* positively regulates *wg* expression in row 5 (reviewed by Perrimon, 1994).

wg is transcribed in neuroectoderm and neuroblasts of row 5, just anterior to *en* and *hh* (Baker, 1987). The secreted Wg protein can be detected anteriorly in row 4 and posteriorly in rows 6/7 (van den Heuvel et al., 1989; González et al., 1991). Loss of *wg* results in non-autonomous defects in both regions: in rows 6/7 there is a fading of *en* and *hh* expression (reviewed in Perrimon, 1994) and a complete loss of row 6 neuroblasts (Chu-LaGraff and Doe, 1993); in row 4 there is failure to express *hkb-lacZ* and a reduced number of neuroblasts (Chu-LaGraff and Doe, 1993).

The *gsb* locus contains two genes, *gsb-distal* (*gsb-d*) and *gsb-proximal* (*gsb-p*), that encode nuclear homeodomain/paired box transcription factors (Bopp et al., 1986). Both *gsb* genes are expressed in neuroectoderm and neuroblasts of row 5 and row 6, as well as in NB 7-1 (Gutjahr et al., 1993; Zhang et al., 1994; Broadus et al., 1995). *gsb* is required to maintain *wg* expression in row 5 (Li and Noll, 1993), and loss of *gsb-d* causes a transformation of row 5 neuroblast identity into row 3/4 neuroblast identity (Zhang et al., 1994; Skeath et al., 1995).

In this study, we are interested in how *hkb* expression is regulated along the anterior/posterior (A/P) axis of the CNS; the mediolateral regulation of *hkb* is beyond the scope of this paper. We find that Hkb is a nuclear protein detected in a subset of neuroectodermal clusters and neuroblasts. We find that the combined function of Wg and Hh accounts for all *hkb* expression in the neuroectoderm: Hh activates *hkb* in rows 1/2 and 7, Wg activates *hkb* in row 4, and both Wg and Hh activate *hkb* in row 5. In addition, we find that Gsb represses *hkb* expression in neuroblasts of rows 5/6 and En represses *hkb* expression in neuroectoderm of rows 6/7. The combined activation and repression mediated by all four segment polarity genes is necessary to establish the normal pattern of *hkb* expression along the A/P axis of the CNS.

MATERIALS AND METHODS

Drosophila strains

The wild-type pattern of Hkb protein was examined in *yellow white* embryos. The following mutant stocks were used: hh^{6N16}/CyO *ftz lacZ* (Mohler, 1988); wg^{CX4}/CyO *ftz lacZ* (Baker, 1987); Df(2R) IIX62/CyO *ftz lacZ* (removes the two *gsb* genes and *zipper;* Lindsley and Zimm, 1992); Df(2R)en^{E}/CyO (removes both *en* and *inv*; Tabata et al., 1995). Standard *Drosophila* genetic techniques were used to make wg^{CX4}, Df(2R) IIX62/CyO *ftz lacZ* and wg^{CX4}/CyO *ftz lacZ*; hh^{6N16}/TM3 *ftz lacZ*. All mutant strains used in this study are null alleles, except hh^{6N16}, which is a strong allele at 25°C.

Antibody production and staining

An N-terminal Hkb peptide (NLHPPQTYSRLFRPWDTQRQC) was synthesized and coupled to keyhole limpet hemacyanin (KLH) for immunization and to bovine serum albumin (BSA) for ELISA screening. Each rat was boosted with 75-100 μg of the KLH-peptide conjugate. Booster immunizations were administered every 3-4 weeks and serum samples were collected 7-10 days post-boost. After six boosts (ELISA-positive response to BSA-peptide conjugate at 1:20,000 dilution), the serum could detect Hkb protein in embryos.

Standard methods were used to fix and stain embryos (Doe, 1992). All embryos were fixed for 20 minutes using PEMFA (100 mM Pipes, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde, pH 6.9). The following primary antibodies were used: rat anti-Hkb serum (1:100); mouse anti-En monoclonal (4D9; 1:5; Patel et al., 1989b); rabbit anti-β-galactosidase serum (1:2000; Cappel). In order to reduce background, sera were pre-absorbed at working concentrations in PBT (PBS, 1% BSA, 0.1% Triton X-100, pH 7.0) against fixed embryos for 2 hours. Histochemical detection of primary antibodies was done using the HRP Vectastain Elite Kit (Vector Labs) according to manufacturer's instructions. HRP was detected using DAB substrate (Pierce). Double labels and detection of β-galactosidase (in order to unambiguously identify mutant embryos; *en* homozygous embryos were picked by lack of detected En/Invected) were performed using direct alkaline phosphatase-conjugated secondary antibodies of the appropriate species (1:200; Southern Biotechnology Associates); alkaline phosphatase detection was done as described by Kania et al. (1990). Embryos were mounted in 85% glycerol, dissected, and viewed on a Zeiss Axioplan microscope. Images were acquired with a Sony DKC-5000 digital camera and figures were assembled in Adobe Photoshop.

RESULTS

Huckebein is a nuclear protein

To determine the subcellular localization of the Hkb protein, and its distribution throughout neurogenesis, we raised an antibody to a Hkb peptide (see Methods). We found that Hkb is a nuclear protein detected in blastoderm termini (Fig. 1A), anterior and posterior midgut primordia (Fig. 1B), salivary gland placodes and procephalon (data not shown) and the ventral CNS (Figs 1C-L, 2). Thus, the Hkb protein pattern closely matches the RNA pattern (Chu-LaGraff et al., 1995), except the protein persists slightly longer than the RNA.

Huckebein is observed in position-specific neuroectodermal clusters and neuroblasts

The spatial pattern of Hkb in the neuroectoderm is complex but highly reproducible (Fig. 1D-F). The neuroectoderm can be divided into seven rows along the A/P axis and three columns along the mediolateral axis; these rows and columns reflect boundaries of gene expression as well as the subsequent arrangement of neuroblasts (Fig. 3; Broadus et al., 1995). At stage 8, Hkb is detected in small clusters of neuroectodermal cells in medial rows 1/2 (Fig. 1D) and intermediate rows 4/5 (Fig. 1D). By stage 9, both clusters have expanded and the row 4/5 cluster now includes a few cells in medial row 5 (Figs 1E, 3A). At stage 10, Hkb levels increase dramatically and expression in rows 1/2 now includes cells in the medial and intermediate columns (Figs 1F, 3B). At late stage 10 or early stage 11, the cluster in rows 1/2 expands anteriorly to include two cells in row 7 (data not shown); although these row 7 cells are contiguous with the intermediate rows 1/2 domain, they can be distinguished by their expression of *en* (data not shown). By late stage 11, Hkb is undetectable in the neuroectoderm (data not shown).

The temporal pattern of Hkb is also highly stereotyped: it is first expressed in a neuroectodermal cluster, then in the neuroblast delaminating at that position, and finally in the early-born progeny of the neuroblast. The following neuroectodermal clusters produce Hkb-positive (Hkb+) neuroblasts: medial rows 1/2 generate NB 2-2; lateral rows 1/2 generate NB 2-4; intermediate row 4 generates NBs 4-2, 4-3, and 4-4; intermediate row 5 generates NB 5-4; and intermediate row 7 generates NB 7-3 (Figs 1G-J, 2). Most, perhaps all, of these Hkb+ neuroblasts produce Hkb+ progeny during their early cell lineages. For example, Hkb is detected in the first progeny of NB 4-2 (GMC 4-2a and the RP2 motoneuron and RP2sib; data not shown). The only exception to the typical temporal progression of *hkb* expression is NB 1-1, which develops from Hkb-negative neuroectoderm and first expresses *hkb* midway through its cell lineage (Fig. 2). Clusters of Hkb+ cells can be detected in the CNS until the end of stage 16 (Fig. 1K,L).

Hedgehog and Wingless proteins activate *huckebein* expression

Hedgehog activates *huckebein* in neuroectoderm of rows 1/2 and 7

hh is transcribed in neuroectoderm of rows 6/7 and the secreted Hh protein can be detected in the adjacent neuroectoderm of rows 5 and 1/2 (Taylor et al., 1993; Tabata and Kornberg, 1994). Because *hkb* is expressed in neuroectoderm adjacent to the Hh domain, we examined embryos homozygous for a strong loss of function *hh* allele (‘*hh* embryos’; see Methods) for changes in the Hkb pattern. We find that Hh activates *hkb* expression in neuroectoderm of rows 1/2, but is not required for *hkb* expression in

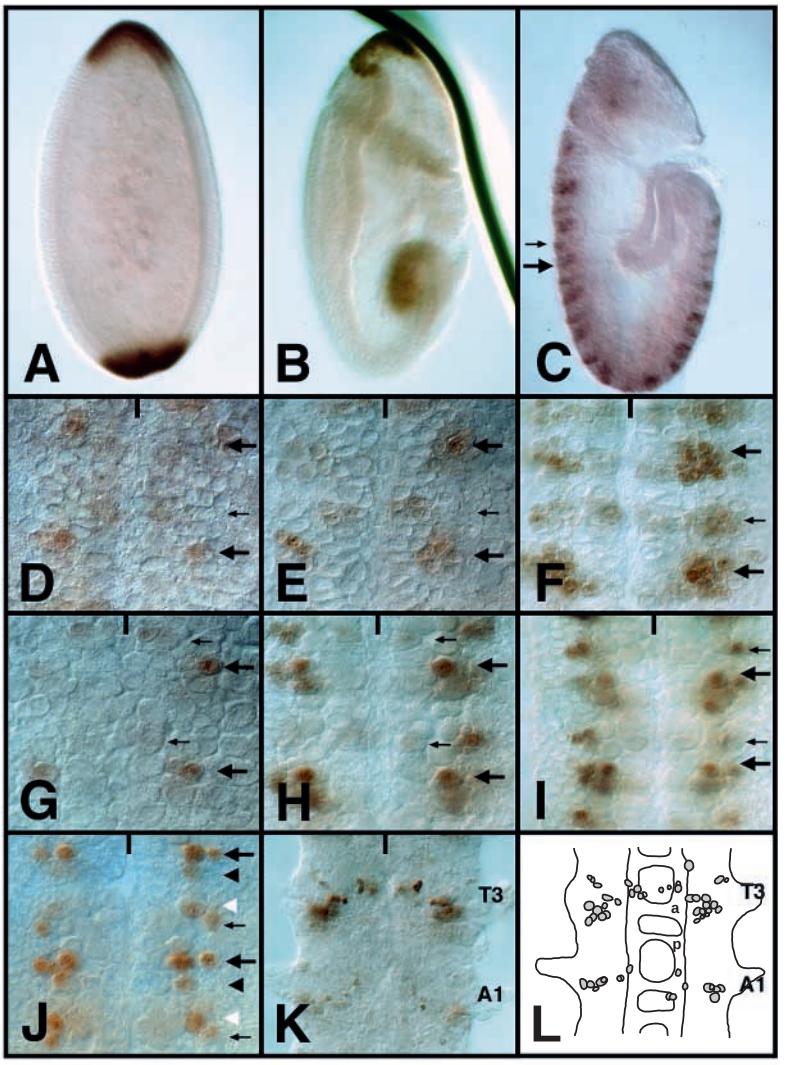

Fig. 1. Hkb protein pattern in wild-type embryos. Neuroblast stages and names according to Broadus et al. (1995); embryonic stages according to Campos-Ortega and Hartenstein (1985). Anterior is up; midline denoted by a black line. (A-C) Lateral views. (A) Blastoderm stage. Hkb is detected in the anterior and posterior termini. (B) Stage 7. Hkb is detected in the anterior and posterior midgut primordia. (C) Stage 10. Hkb is observed in segmentally repeated clusters of neuroectodermal cells in rows 1/2 (small arrow) and row 4 (large arrow). (D-F) Ventral view of two segments of neuroectoderm. (D) Late stage 8 and (E) stage 9. Hkb is detected in medial rows 1/2 (small arrow) and intermediate row 4 (large arrow), and by stage 9 low levels of Hkb are visible in the medial and intermediate row 5. (F) Stage 10. In rows 1/2, Hkb is now detected in a medial and intermediate cluster (small arrow), which will include two En+ cells of row 7 at late stage 10 (data not shown). In rows 4/5, Hkb is present at high levels at the intermediate position and at low levels in the medial row 5 position (large arrow). (G-J) Ventral view of neuroblasts. (G) Stage 9 and (H) stage 10. Hkb is detected in low levels in NB 2-2 (small arrow) and at high levels in NB 4-2 (large arrow). (I) Stage 11. Hkb is observed in NB 1-1 (not shown), NB 2-4 (small arrow), NB 4-2 (large arrow), NB 4-4 (large arrow, lateral to NB 4-2), and NB 5-4 (not shown). (J) Late stage 11. Hkb is detected in NB 1-1 (not shown), NB 2-4 (small arrow), NB 4-2 (out of focal plane), NB 4-3 (large black arrow, medial to NB 4-4), NB 4-4 (large arrow), NB 5-4 (black triangle), and NB 7-3 (white triangle). (K) Dorsal view of the third thoracic (T3) and first abdominal (A1) segments in a stage 16 dissected nerve cord. Hkb is detected at higher levels and in more cells in the thorax. (L) Camera lucida tracing of the CNS in K. The neuropil is outlined; Hkb+ cells, gray; a, anterior commissure; p, posterior commissure.

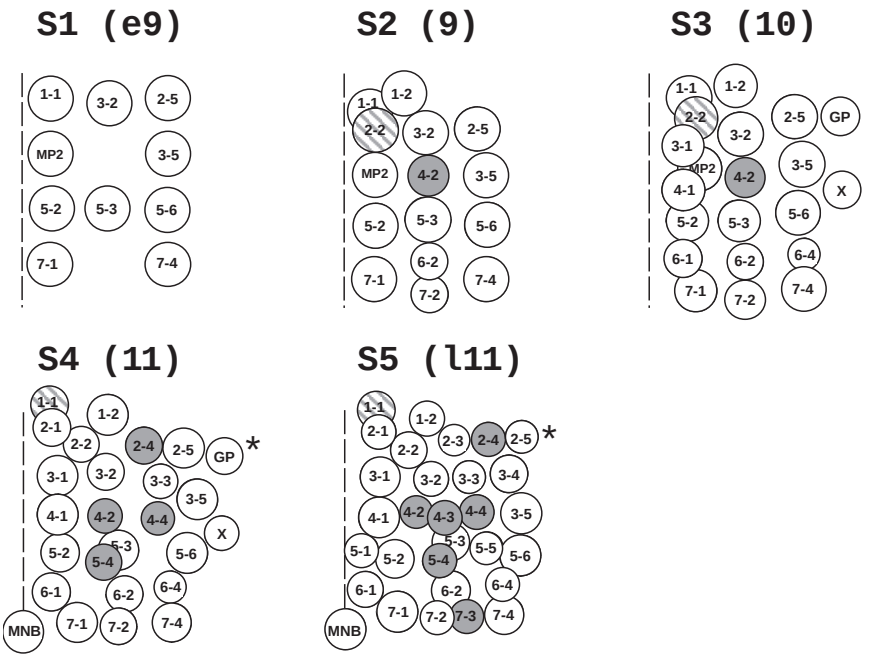

Fig. 2. Hkb protein is detected in eight neuroblasts per hemisegment in wild-type embryos. All Hkb+ neuroblasts delaminate from Hkb+ neuroectoderm, with the exception of NB 1-1, which becomes Hkb+ midway through its cell lineage (see text for details). Hatched cells, low Hkb; gray cells, high Hkb. Unlike Broadus et al. (1995), we find that NB 5-4 forms slightly ventral to NB 5-3 and can be on either side. Embryonic stages, Campos-Ortega and Hartenstein (1985); neuroblast names and stages, Broadus et al. (1995); anterior is up; ventral midline, dotted line; tracheal pits, asterisk.

row 5 (Figs 3, 4B, 5B, 6B). At late stage 10/early stage 11, Hh also activates *hkb* expression in a small cluster of row 7 neuroectodermal cells that produces the Hkb+ NB 7-3 (data not shown); NB 7-3 occasionally lacks Hkb protein in *hh* embryos (data not shown). In addition, we find that the post-S1 row 2 neuroblasts fail to form in *hh* embryos (data not shown). Thus, Hh acts non-autonomously in the posterior direction (from rows 6/7 into rows 1/2) to activate *hkb* expression; Hh also acts within row 7 to trigger *hkb* expression in the intermediate neuroectoderm of row 7.

Two domains of *hkb* expression persist in *hh* embryos: neuroectoderm of rows 4/5 and NB 1-1. This suggests that there are other mechanisms for activating *hkb* expression (see below). In addition, Hh is produced in rows 6/7, yet the majority of these cells do not express *hkb*. This suggests that there are repressors that block Hh activation of *hkb* expression in rows 6/7 (see below).

Wingless activates *huckebein* in neuroectoderm of row 4

wg is transcribed in neuroectoderm of row 5 and the secreted Wg protein can be detected in the adjacent neuroectoderm of rows 4 and 6/7 (van den Heuvel et al., 1989; González et al., 1991). In embryos homozygous for a null *wg* allele (‘*wg* embryos’; see Methods), Hkb is never detected in row 4 neuroectoderm (Figs 3, 4C, 5C) or neuroblasts (Fig. 6C). We conclude that Wg non-autonomously activates *hkb* in neuroectoderm and neuroblasts of row 4. In addition to loss of Hkb in *wg* embryos, we observe ectopic Hkb in rows 6/7 late in stage 10 (Figs 3B, 5C,F). This ectopic Hkb is likely due to the loss of *en* expression in rows 6/7 in *wg* embryos (Bejsovec and Wieschaus, 1993; van den Heuvel et al., 1993); *en* encodes a repressor of *hkb* expression (see below).

Three domains of *hkb* expression persist in *wg* embryos: neuroectoderm of rows 1/2, neuroectoderm of row 5, and NB 1-1 (Figs 3, 5C, data not shown). Rows 1/2 expression is induced by Hh (see above). However, based on single mutant analysis, we cannot distinguish whether *hkb* expression in neuroectoderm of row 5 and NB 1-1 is (a) independent of both Hh and Wg or (b) redundantly activated by either Wg or Hh. The latter model is most likely for row 5, which is exposed to both Wg and Hh signals.

Wingless or Hedgehog can activate *huckebein* in neuroectoderm of row 5, but not in NB 1-1

To determine the role of Wg and Hh function in activating *hkb* expression in neuroectoderm of row 5 and NB 1-1, we examined embryos lacking both *wg* and *hh* function (‘*wg*; *hh* embryos’; see Methods). In *wg*; *hh* embryos, Hkb is not observed in neuroectoderm of row 5 (Figs 3, 4D, 5D), but is still detected in NB 1-1 (Fig. 6G). Thus, Wg and Hh can each activate *hkb* expression in row 5, but neither is required for expression in NB 1-1.

These data, taken together with results in the previous sections, show that the CNS expression of Hkb is activated by three different mechanisms: (1) activated by Hh in rows 1/2, 5 and 7; (2) activated by Wg in rows 4 and 5; and (3) activated by a Hh- and Wg-independent mechanism during the lineage of NB 1-1.

Gooseberry represses *huckebein* expression in row 5 neuroblasts

Early-forming neuroblasts of rows 5 and 6 never express *hkb* (Figs 1, 2, 6A,H), even though they develop from Hkb+ neuroectoderm (row 5), and/or are exposed to Wg and Hh (rows 5 and 6). What prevents Wg- and Hh-mediated activation of *hkb* in neuroblasts of rows 5 and 6 during the early stages of neurogenesis? Good candidates for repressing *hkb* activation are the tandem *gsb* genes, which encode nuclear Pax-type proteins detected in neuroectoderm and neuroblasts of rows 5 and 6 (Doe, 1992; Gutjahr et al., 1993; Zhang et al., 1994). In embryos lacking both *gsb-d* and *gsb-p* (‘*gsb* embryos’; see Methods), *hkb* is ectopically expressed in an intermediate row 5 neuroblast (nuclear signal in NB 5-3; Fig. 6D), and in

several intermediate row 6 neuroblasts (Fig. 6I). Restriction of ectopic Hkb to the intermediate column neuroblasts may reflect the normal mediolateral restriction of high-level *hkb* expression to the intermediate column neuroectoderm of rows 4/5 (Fig. 1D-F). In *gsb* embryos, the neuroectodermal Hkb pattern matches wild-type embryos at all stages, with *hkb* expressed in row 5 but not in row 6 (Figs 4A,E, 5A,E). We conclude that *gsb* represses *hkb* expression in intermediate row 5 and 6 neuroblasts, but does not regulate neuroectodermal *hkb* expression.

The ectopic *hkb* expression in row 5 and 6 neuroblasts in *gsb* embryos could be Wg-dependent, Hh-dependent, or independent of both (e.g. similar to NB 1-1). We find that in *wg*, *gsb* embryos, NB 5-3 is Hkb-negative (Fig. 6E), even though it is exposed to Hh signal. We are unable to score *hkb* expression in row 6 neuroblasts because they do not form in the absence of *wg* (Chu-LaGraff and Doe, 1993). These results strongly suggest that (1) Gsb blocks Wg-dependent activation of *hkb* in NB 5-3 (and possibly in row 6 neuroblasts); and (2) Hh is not competent to activate *hkb* in NB 5-3, despite its ability to activate *hkb* expression in neuroectoderm of row 5.

Engrailed represses *huckebein* expression in rows 6/7 neuroectoderm

In wild-type embryos, *hkb* is not expressed in most of the neuroectoderm and neuroblasts found in rows 6/7, even though these cells are exposed to both Wg and Hh (Figs 4A, 5A, 6A,G). What blocks *hkb* expression in rows 6 and 7? A good candidate is *en*, which encodes a transcription factor detected in all neuroectoderm and neuroblasts of rows 6 and 7 (DiNardo et al., 1985; Doe, 1992). Because *invected* (*inv*) plays a functionally redundant role with *en* (Gustavson et al., 1996), we examined embryos lacking both *en* and *inv* (‘*en* embryos’; see Methods). We find that in stage 9 *en* embryos, the cluster of Hkb+ cells in medial rows 1/2 has expanded anteriorly into rows 6/7 (Figs 3A, 4G), with some variablity amongst hemisegments in the number of cells expressing ectopic Hkb. Similarly, in stage 10 *en* embryos, the cluster of Hkb+ cells in intermediate rows 1/2 has expanded anteriorly into rows 6/7 (Figs 3B, 5G). However, in some hemisegments there is a loss of *hkb* expression in rows 1/2 and 6/7, probably due to loss of Hh in *en* embryos at this stage (Tabata et al., 1992; Lee et al., 1992). The mediolateral restriction of ectopic Hkb in rows 6/7 exactly matches regulation of the Hkb pattern in rows 1/2, indicating that the same (unknown) mediolateral regulator(s) control *hkb* expression in rows 1/2 and 6/7. We conclude that *en* represses *hkb* in neuroectoderm of rows 6 and 7. Rows 6/7 neuroectoderm is exposed to both Wg and Hh activating signals, and thus it is likely that *en* can block both signaling pathways (see Discussion).

In wild-type embryos, *hkb* and *en* are coexpressed in NB 7-3 (Broadus et al., 1995; Lundell et al., 1996), thus *en* does not always repress *hkb*. Furthermore, in *en* embryos there is no ectopic *hkb* expression in row 6/7 neuroblasts, and in *gsb* embryos there is coexpression of *hkb* and *en* in several row 6 neuroblasts. Thus, in contrast to its role in the neuroectoderm, apparently *en* has no function in repressing *hkb* in neuroblasts.

DISCUSSION

We have generated an antibody to a Hkb peptide, and find that the Hkb protein is predominantly localized to the nucleus in all

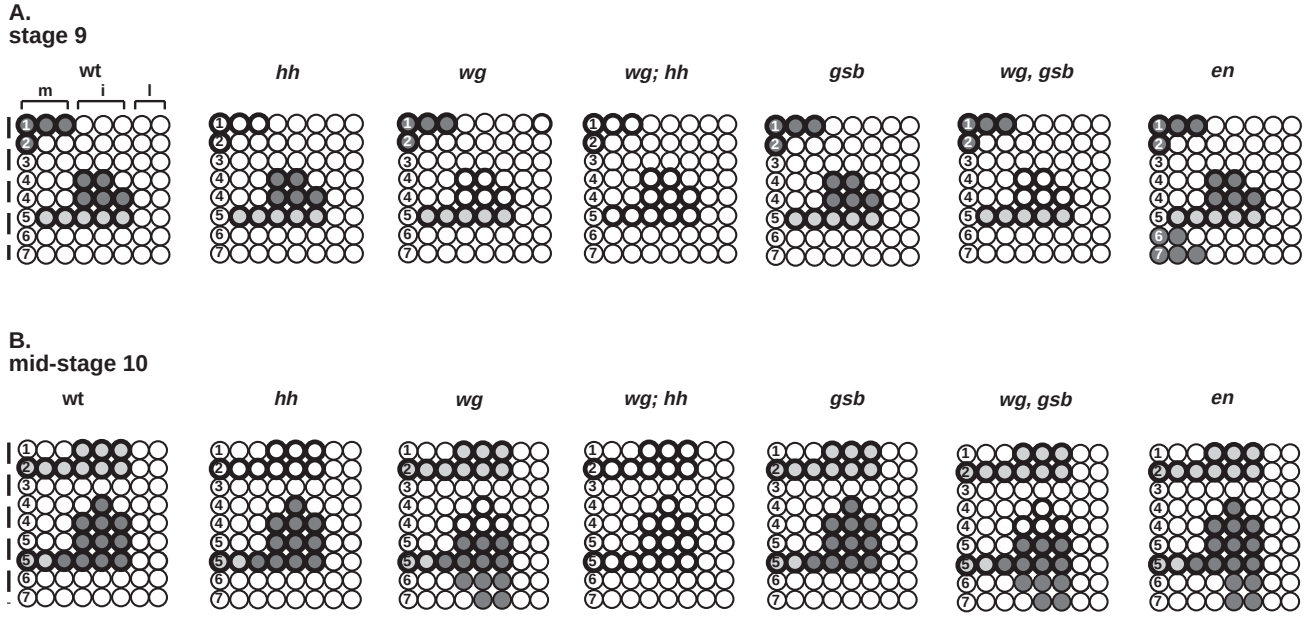

Fig. 3. Summary of Hkb protein patterns in the neuroectoderm at stage 9 (A) and mid-stage 10 (B) in the following genotypes (from left to right): wild type, *hh*, *wg*, *wg hh* double mutant, *gsb*, *wg gsb* double mutant, and *en*. For A, see text and Fig. 4 for details. For B, see text and Fig. 5 for details. Bold outlined cells are Hkb+ in wild-type embryos; actual Hkb+ cells are depicted in dark gray (high levels) or light gray (low levels). Although in wild-type embryos row 7 is Hkb+, this expression is only detected at late stage 10/early stage 11. One hemisegment of neuroectoderm is shown. Anterior is up; dotted line, midline; m, medial column; i, intermediate column; l, lateral column; numbers indicate rows.

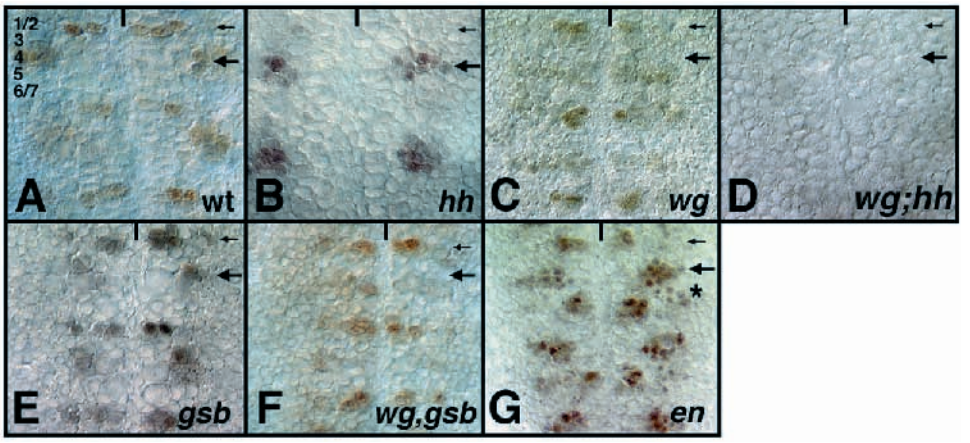

Fig. 4. Hkb protein pattern in the neuroectoderm of stage 9 wild-type (A), *hh* (B), *wg* (C), *wg hh* double mutant (D), *gsb* (E), *wg gsb* double mutant (F) and *en* (G) embryos. Ventral views of two segments are shown. Anterior is up; midline, line; numbers in A indicate rows; rows 1/2, small arrow; row 4, large arrow. (A) In wild-type embryos, Hkb is detected in rows 1/2, 4, and 5. (B) In *hh* embryos, Hkb is normal in rows 4 and 5, but is not detected in the rows 1/2 cluster (96% of hemisegments scored, *n*=168). In a small fraction of hemisegments there are a few scattered cells that are Hkb+ in rows 1/2 (data not shown). (C) In *wg* embryos, Hkb is normal in rows 1/2 and 5, but is not observed in row 4 (*n*≥99 hemisegments). (D) In *wg; hh* embryos, Hkb is not detected in any portion of the neuroectoderm (*n*≥60 hemisegments). (E) In *gsb* embryos, Hkb is identical to wild type at this stage. (F) In *wg, gsb* embryos, Hkb is detected in the rows 1/2 and 5 clusters, but not in row 4 (*n*≥74 hemisegments). This is identical to *wg* embryos. (G) In *en* embryos, Hkb is detected in rows 1/2, 4 and 5 as in wild-type embryos, but ectopic Hkb is observed in approximately two to three medial row 6 and 7 cells (asterisk) anterior to the rows 1/2 cluster (67% of hemisegments scored, *n*=113). The numbers of cells with ectopic Hkb expression is variable from hemisegment to hemisegment.

embryonic tissues in which it is expressed. Hkb may regulate transcription in the CNS, since *hkb* encodes a putative DNA binding protein (Brönner et al., 1994) and mutations in *hkb* result in altered gene expression in neurons and glia, including loss of *eve* expression in the RP2 neuron (Chu-LaGraff et al., 1995) and loss of *repo* expression in the A and B glia (Bossing et al., 1996a). We find that two evolutionarily conserved signaling molecules, Wg and Hh, both function non-autonomously to activate *hkb* expression in specific domains within the neuroectoderm, whereas the nuclear Gsb and En proteins repress *hkb* expression. Thus, the stereotyped pattern of *hkb* in the CNS, which is essential for normal CNS development, is established by activating input from both the Wg and Hh signaling pathways and repression by the Gsb and En transcription factors.

Hedgehog and Wingless activate *huckebein* expression in specific domains of neuroectoderm and neuroblasts

Hh is expressed in rows 6/7 and signals in the posterior direction to activate *hkb* expression in neuroectoderm of rows 1/2 and to trigger the formation of the post-S1 row 2 neuroblasts. Expression of *hkb* in rows 1/2 is necessary for normal CNS development, since NB 2-2 requires *hkb* to produce interneurons and motoneurons with normal axon trajectories (Bossing et al., 1996a). The neuroectoderm of rows 6/7 receives the Hh signal, and a pair of cells in row 7 normally express *hkb*, as does the NB 7-3 that forms at this position. Loss of Hh results in partial loss of Hkb in NB 7-3. Consistent with this result, Patel et al. (1989a) observe a loss of serotonergic neurons in *hh* embryos; these neurons derive from NB 7-

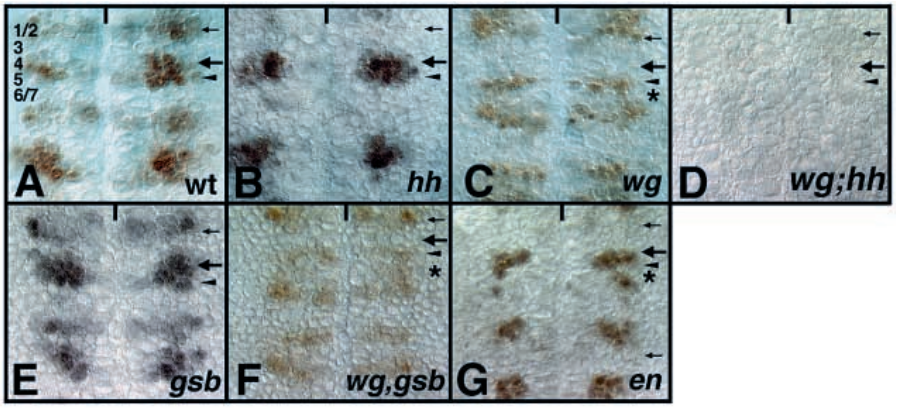

Fig. 5. Hkb protein pattern in the neuroectoderm of stage 10 wild-type (A), *hh* (B), *wg* (C), *wg hh* double mutant (D), *gsb* (E), *wg gsb* double mutant (F) and *en* (G) embryos. Ventral view of two segments are shown in each panel. Anterior is up; midline, line; numbers in A indicate rows; rows 1/2, small arrow; row 4, large arrow; row 5, arrowhead; ectopic rows 6/7, asterisk. (A) In wild-type embryos, Hkb is detected in rows 1/2, 4 and 5. (B) In *hh* embryos, Hkb is normal in rows 4 and 5, but is not detected in rows 1/2. Hkb protein in rows 4 and 5 fades early at late stage 10 (data not shown); the row 4 phenotype is presumably due to loss of Wg in *hh* embryos at stage 10 (Bejsovec and Wieschaus, 1993; van den Heuvel et al., 1993), whereas the row 5 phenotype is probably due to the loss of both *hh* and *wg* at this stage (see Discussion). (C) In *wg* embryos, Hkb is observed in rows 1/2 and 5, but never in row 4. Ectopic Hkb protein is detected in rows 6/7, presumably due to loss of *en* expression (see Discussion). (D) In *wg; hh* embryos, Hkb is not detected in any portion of the neuroectoderm (although Hkb persists in the salivary gland placodes and NB 1-1). (E) In *gsb* embryos, Hkb is identical to wild type. (F) In *wg, gsb* embryos, Hkb is normal in rows 1/2 and 5, but is not detected in row 4, and there is ectopic Hkb in rows 6/7. This is identical to *wg* embryos. (G) In *en* embryos, Hkb is normal in rows 1/2, 4 and 5, but ectopic Hkb protein is detected in rows 6/7. However, in some hemisegments, rows 1/2 and 6/7 Hkb expression is extinguished (bottom small arrow), probably due to loss of Hh at this stage (Tabata et al., 1992; Lee et al., 1992).

3 (Lundell et al., 1996). The partial phenotype of *hh* in activating row 7 *hkb* expression suggests that a second gene (possibly *wg*) contributes to *hkb* expression in this region; alternatively, the *hh* allele used provides enough Hh activity to trigger some row 7 *hkb* expression.

wg is required nonautonomously to activate row 4 expression of *lacZ* from an enhancer trap insertion at the *hkb* locus (Chu-LaGraff and Doe, 1993). Here we show that *wg* activates expression of the native *hkb* gene in neuroectoderm and neuroblasts of row 4. The same phenotype is observed in embryos lacking maternal and zygotic expression of the *porcupine*, *dishevelled* and *armadillo* genes in the '*wingless* signaling pathway' (J. A. M. et al., unpublished results). It is not known if the *wg* pathway directly activates *hkb* in row 4 neuroblasts, or whether neuroblast expression is independently maintained following activation of *hkb* in row 4 neuroectoderm.

hkb is expressed in the neuroectoderm of row 5 and in one row 5 neuroblast (NB 5-4). Row 5 expression of *hkb* (just anterior to the Hh domain) is virtually unaffected by loss of *hh*. This domain of expression is also normal in *wg* embryos, despite high levels of Wg in row 5. However, *hkb* expression in row 5 is missing in embryos doubly mutant for *wg* and *hh*. Thus, Wg and Hh are both able to activate *hkb* in this region, most likely via different pathways (Fig. 7).

Wg and Hh are necessary to activate *hkb* in the embryonic CNS, with the notable exception of NB 1-1. This neuroblast develops from the neuroectoderm of rows 1/2 just posterior to the domain of *hh* expression; it forms prior to Hkb expression in the neuroectoderm, and only expresses *hkb* midway through its cell lineage. This 'sublineage-specific' expression of *hkb* in NB 1-1 is different from all other Hkb+ neuroblasts, which develop from Hkb+ neuroectoderm, and is independent of *hh* and *wg*. This indicates that a third mechanism, in addition to Hh and Wg, can activate *hkb* expression (Fig. 7).

Engrailed and Gooseberry repress *huckebein* expression

Although most of the neuroectoderm is exposed to either Wg or Hh signals, only a small subset of cells in each segment expresses *hkb*. In areas that *hkb* is not expressed, these domains are either unable to respond to positive signals due to the presence of repressors, or are not exposed to positive regulators.

Gsb is expressed in the neuroectoderm and neuroblasts of rows 5 and 6; it represses *hkb* expression in row 5 and 6 neuroblasts. For example, the intermediate row 5 neuroblast, NB 5-3, is normally Gsb+ and Hkb-negative. In the absence of Gsb, this row 5 neuroblast becomes Hkb+, similar to the adjacent NB 4-2. The Hkb+ 'NB 5-3' is probably transformed into the NB 4-2 identity, since one marker for the NB 4-2 lineage, the Eve+ RP2 neuron, is duplicated in *gsb* embryos (Patel et al., 1989a; Bhat, 1996). Gsb represses row 5 *hkb* expression by antagonizing autocrine Wg signaling in row 5 neuroblasts; in *wg*, *gsb* double mutant embryos, NB 5-3 does not express *hkb*. This is further confirmed by the result that in *wg*; *gsb* embryos the Eve+ RP2 neuron is missing (Bhat, 1996; N. H. Patel, personal communication). The *gsb* genes therefore normally act to repress Wg activation of *hkb* expression in NB 5-3, allowing NB 5-3 development and preventing the formation of a duplicate NB 4-2 (Fig. 7). In contrast to row 5 neuroblasts, the neuroectoderm of row 5 expresses both *hkb* and *gsb* (although the *hkb* expression is rather weak). This suggests that *gsb* represses *hkb* completely in neuroblasts but weakly or not at all in neuroectoderm. One explanation might

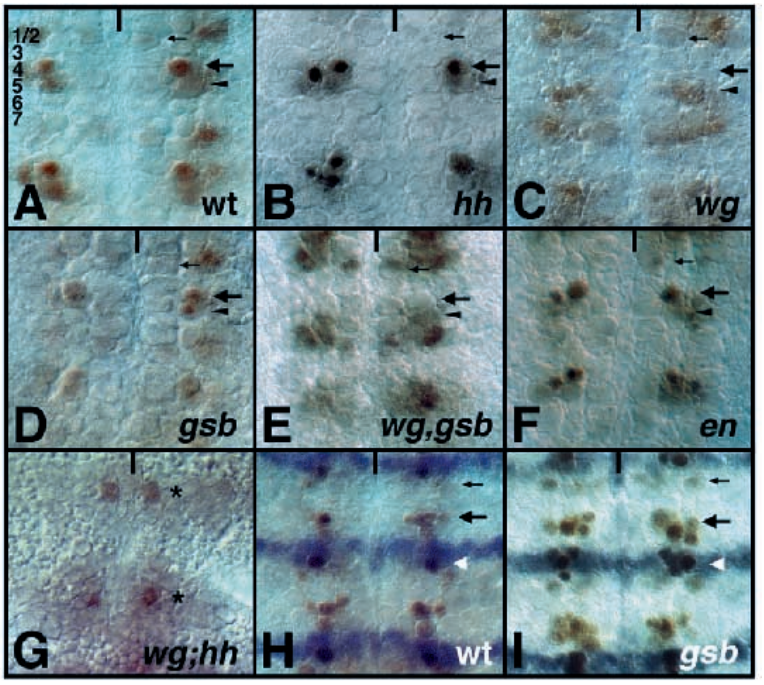

Fig. 6. Hkb protein pattern in neuroblasts of stage 10 (A-F) and 11 (G-I) wild-type (A,H), *hh* (B), *wg* (C), *gsb* (D,I), *wg gsb* double mutant (E), *en* (F), and *wg hh* double mutant (G) embryos. Ventral view of two segments are shown in each panel. Anterior is up; midline, line; numbers in A indicate rows; NB 1-1, asterisk; NB 2-2, small arrow; NB 4-2, large arrow; NB 5-3, arrowhead; NB 7-3, white triangle. (A) In wild-type embryos, Hkb is detected in NB 2-2 and NB 4-2, but not in NB 5-3; it should be noted that although there appears to be faint, non-nuclear staining in NB 5-3, this is bleed-through from the heavily stained rows 4/5 neuroectoderm (see Figs 1F, 5A). (B) In *hh* embryos, Hkb is detected in NB 4-2; NB 2-2 (small arrow) and NB 2-4 (not shown) do not form and Hkb is not detected at these positions. By stage 11, NB 7-3 occasionally lacks Hkb protein (23% of hemisegments, *n*=78, data not shown). (C) In *wg* embryos, Hkb is detected in NB 2-2, but never in NB 4-2 (NB 4-2 forms in 19% of hemisegments scored; *n*=48). (D) In *gsb* embryos, ectopic Hkb is observed in the nucleus of NB 5-3 (73%, *n*=108 hemisegments), in addition to the normal Hkb+ NBs 2-2 and 4-2. (E) In *wg; gsb* embryos, like *wg* embryos, Hkb is detected in NB 2-2, but not in NB 4-2 (NB 4-2 formed in 26% of hemisegments; *n*=53) or in NB 5-3 (99% of hemisegments, *n*=72). This shows that Wg is necessary for Hkb expression in NB 5-3 in *gsb* embryos (see D). (F) In *en* embryos, Hkb has a wild-type pattern in neuroblasts, including NB 7-3 (data not shown). (G) In *wg; hh* embryos, neuroblasts have no detectable Hkb (out of the plane of focus), with the notable exception of NB 1-1 (asterisk; compare to wild type in Fig. 2; Chu-LaGraff et al., 1995, and Bossing et al., 1996). (H) Wild-type embryo labeled for Hkb (brown) and En (purple). Both Hkb and En are detected in NB 7-3, making the cell appear black. (I) *gsb* embryo labeled for Hkb (brown) and En (purple). In addition to NB 7-3, several row 6 neuroblasts are both Hkb+ and En+ (76%, *n*=63 hemisegments); two are shown.

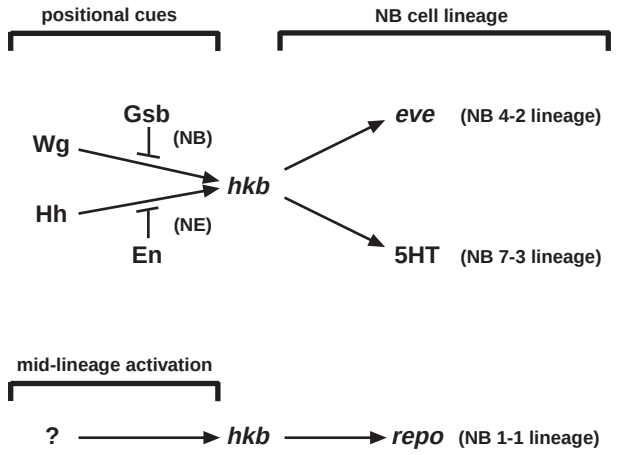

Fig. 7. *hkb* translates neuroectodermal positional cues into neuroblast-specific development. Wg and Hh activate *hkb* in the neuroectoderm (see text). Gsb represses Wg activation of *hkb*: *gsb* embryos show Wg-dependent ectopic *hkb* in row 5 and 6 neuroblasts. En blocks Hh activation of *hkb*: ectopic *hkb* is observed in neuroectoderm of rows 6/7 in *wg* embryos at stage 10, when *en*, but not *hh*, is extinguished (Tabata et al., 1992; Bejsovec and Wieschaus, 1993; van den Heuvel, 1993). It is unknown if En blocks Wg activation of *hkb*: although ectopic *hkb* is not detected in *hh* embryos, both *en* and *wg* are extinguished at stage 10 (Bejsovec and Wieschaus, 1993). Position-specific expression of *hkb* is required for *eve* expression, axon pathfinding, and target recognition in the RP2 neuron in the NB 4-2 lineage (Chu-LaGraff et al., 1995) and for production of the neurotransmitter serotonin (5HT) in neurons derived from the NB 7-3 lineage (Lundell et al., 1996). The mid-lineage activator of *hkb* in NB 1-1 is unknown; *hkb* is required for *repo* expression in the A and B glia derived from the NB 1-1 lineage (Bossing et al., 1996a). NE, neuroectoderm; NB, neuroblast.

be that row 5 neuroectoderm is exposed to much higher levels of Wg and Hh activators than are the neuroblasts.

Consistent with its role in row 5 neuroblasts, *gsb* is also required to block *hkb* expression in row 6 neuroblasts. In *gsb* embryos, ectopic Hkb is detected in row 6 neuroblasts. The new Hkb+ row 6 neuroblasts coexpress En and thus are molecularly similar to the normally Hkb+ and En+ NB 7-3. In fact, at least one of these row 6 neuroblasts has probably been transformed into a duplicate NB 7-3 fate, as judged by the duplication of a NB 7-3 lineage marker (serotonergic neurons; Lundell et al., 1996) in *gsb* embryos (Patel et al., 1989a). We do not know if ectopic *hkb* expression in row 6 neuroblasts is a result of Wg or Hh signaling, because row 6 neuroblasts do not form in *wg, gsb* embryos (similar to *wg* embryos; Chu-LaGraff and Doe, 1993), and we have not examined *gsb*; *hh* embryos. Finally, we see no derepression of *hkb* in neuroectoderm of row 6 in *gsb* embryos; either Gsb does not repress *hkb* in the neuroectoderm of row 6 (similar to row 5) or perhaps both Gsb and En act redundantly to repress *hkb* in row 6.

En is expressed in the neuroectoderm and neuroblasts of rows 6/7. In *en* embryos, there is a stage-dependent derepression of *hkb* expression in the medial or intermediate column neuroectoderm of rows 6/7 (indicating that stage-specific mediolateral regulation persists in the absence of *en*). However, the requirement for *en* to repress *hkb* expression is not complete and can be quite variable, suggesting that another mechanism might contribute to repression of *hkb* in rows 6/7. In stage 10 *wg* and *wg, gsb* embryos, *en* expression is lost (Bejsovec and Wieschaus, 1993; van den Heuvel et al., 1993), but we still observe ectopic *hkb* in neuroectoderm of rows 6 and 7. We conclude that Hh is responsible for ectopic rows 6/7 expression of *hkb* in *en* embryos, and that the normal function of En is to block Hh-mediated activation of *hkb* expression in the neuroectoderm (Fig. 7). Surprisingly, although *en* is expressed in all neuroblasts of rows 6/7, *en* embryos show no derepression of *hkb* in neuroblasts. Thus, En represses *hkb* expression in neuroectoderm but not in neuroblasts.

In wild-type embryos, Hkb protein is not detected in the neuroectoderm of row 3. Row 3 is the region furthest from both Wg (row 5) and Hh (rows 6/7) signals, and may be out of range of both signaling proteins. Perhaps a threshold level of Wg and/or Hh is required to activate *hkb*. Alternatively, another gene expressed in row 3 may block *hkb* expression. The former hypothesis is supported by recent results of Bhat (1996), who showed that misexpression of *wg* in row 3 resulted in ectopic *hkb-lacZ* expression and duplication of the Eve+ RP2 neuron. Thus, row 3 is competent to express Hkb, but receives insufficient Wg (and perhaps Hh) signal to activate Hkb.

Mediolateral regulation of *huckebein* expression

The pattern of *hkb* along the mediolateral axis is just as complex as the regulation along the A/P axis. For example, the Hkb+ row 4 cluster is observed only in the intermediate column, never in the medial or lateral columns; the rows 1/2 cluster is initially medial, but extends to the intermediate column with time. We have shown that Wg and Hh are responsible for all *hkb* expression in the neuroectoderm, which suggests that mediolateral regulation involves repression of *hkb* expression. Candidates for mediolateral repression of *hkb* in the neuroectoderm include the *spitz* group genes and *ventral nervous system defective*, which are expressed in medial domains (Golembo et al., 1996; Jiménez et al., 1995; Mellerick and Nirenberg, 1995).

In the future, it will be informative to identify the *hkb cis*-regulatory sequences that mediate each of the positional inputs. This might include distinct elements required for Hh activation, Wg activation, En repression, Gsb repression, mediolateral repression, and sublineage-dependent activation in NB 1-1. Integrating these diverse positional inputs is required to establish the normal pattern of *hkb* in the neuroectoderm, which is subsequently required for the development of specific neuroblast cell lineages.

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