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

Jocelyn A. McDonald and Chris Q. Doe*
Howard Hughes Medical Institute, Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801, USA

*Author for correspondence

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. 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 know 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.

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). 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).
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; Brönnert 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 – en(Sey) (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: hh618CyO ftz lacZ (Mohler, 1988); wgCX4 CyO ftz lacZ (Baker, 1987); Df(2R) IIX62 CyO ftz lacZ (removes the two gsb genes and zipper; Lindsley and Zimm, 1992); Df(2R)enE4CyO (removes both en and inv; Tabata et al., 1995). Standard Drosophila genetic techniques were used to make wgCX4, Df(2R) IIX62 CyO ftz lacZ and wgCX4 CyO ftz lacZ; hh618TM3 ftz lacZ. All mutant strains used in this study are null alleles, except hh618, which is a strong allele at 25°C.

Antibody production and staining

An N-terminal Hkb peptide (NLHPQTYSRLFRPWDTRQQC) was synthesized and coupled to keyhole limpet hemocyanin (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 MgSO4, 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.
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 injected (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 variability 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 elaborated regions of the neural tissue.
embryonic tissues in which it is expressed. Hkb may regulate transcription in the CNS, since hkb encodes a putative DNA binding protein (Börnner 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 hkb embryos; these neurons derive from NB 7-
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), 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 nuclei of NB 5-3 (73%, n=108 hemisegments), in addition to the normal Hkb+ NBs 2-2 and 4-2. (E) In 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 gsb 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.
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.

We thank Jim Skeath for comments on the manuscript; S. Miklasz of the Immunological Resource Center of the University of Illinois and Q. Chu-LaGraff for help in producing the Hkb antiserum; T. Orenic for fly stocks; and N. H. Patel for antibodies and sharing unpublished results. This work was supported by the NIH (27056). C. Q. D. is an Assistant Investigator of the Howard Hughes Medical Institute.

REFERENCES


Bhat, K. M. (1996). The patched signaling pathway mediates repression of...
Chu-LaGraff, Q. and Doe, C. Q.


(accepted 26 December 1996)