The *Drosophila* neurogenic gene *big brain*, which encodes a membrane-associated protein, acts cell autonomously and can act synergistically with *Notch* and *Delta*

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**SUMMARY**

In the developing nervous system of *Drosophila*, cells in each proneural cluster choose between neural and epidermal cell fates. The neurogenic genes mediate the cell-cell communication process whereby one cell adopts the neural cell fate and prevents other cells in the cluster from becoming neural. In the absence of neurogenic gene function, most, if not all of the cells become neural. *big brain* is a neurogenic gene that encodes a protein with sequence similarity to known channel proteins. It is unique among the neurogenic genes in that previous genetic studies have not revealed any interaction between *big brain* and the other neurogenic genes. Furthermore, the neural hypertrophy in *big brain* mutant embryos is less severe than that in embryos mutant for other neurogenic genes.

In this paper, we show by antibody staining that *bib* is expressed in tissues that give rise to neural precursors and in other tissues that are affected by loss of neurogenic gene function. By immunoelectron microscopy, we found that *bib* is associated with the plasma membrane and concentrated in apical adherens junctions as well as in small cytoplasmic vesicles. Using mosaic analysis in the adult, we demonstrate that *big brain* activity is required autonomously in epidermal precursors to prevent neural development. Finally, we demonstrate that ectopically expressed *big brain* acts synergistically with ectopically expressed *Delta* and *Notch*, providing the first evidence that *big brain* may function by augmenting the activity of the Delta-Notch pathway. These results are consistent with *bib* acting as a channel protein in proneural cluster cells that adopt the epidermal cell fate, and serving a necessary function in the response of these cells to the lateral inhibition signal.

Key words: *big brain*, *Notch*, *Delta*, neurogenesis, lateral inhibition, *Drosophila*, genetic interaction

**INTRODUCTION**

The first step in the development of the *Drosophila* nervous system is the specification of neural precursors (NPs) at precise locations. Groups of uncommitted ectodermal cells at particular positions acquire the potential to become NPs through expression of proneural bHLH transcription factors, which include the *achaete-scute* complex and *atonal* gene products. Within each of these groups of cells, called proneural clusters, a small subset of cells go on to express elevated levels of the proneural genes. Finally, proneural gene expression is restricted within each cluster to a single cell, which adopts the neural fate; the rest of the proneural cluster cells stop expressing the proneural genes and adopt the epidermal fate. Loss of proneural gene function results in decreased numbers of NPs, while gain of function results in increased numbers of NPs (reviewed in Ghysen et al., 1993).

Experiments in the grasshopper have shown that cell-cell interactions are required for specifying single NPs within each cluster (Doe and Goodman, 1985). When the NP is ablated, another cell from the proneural cluster replaces it. It thus appears that the presence of the original NP prevents the other cells from becoming NPs, through a process called lateral inhibition. A variety of experiments in *Drosophila* support this lateral inhibition model (for review see Simpson, 1990). The concept of lateral inhibition has been extended to include the process in which cells of the proneural cluster compete with each other through cell-cell interactions, eventually selecting a single NP (Goriely et al., 1991). Accordingly, each cell inhibits the ability of the other cells to become neural while increasing its own neural potential. This situation is unstable and, once one cell gains an advantage, it quickly adopts the neural fate, forcing all the other cells to become epidermal.

Mutations in a number of genes, known as the neurogenic genes, disrupt the lateral inhibition process, allowing many more cells to adopt the neural cell fate than in the wild-type situation (Lehmann et al., 1983). The neurogenic genes are also required for the development of other tissues including somatic muscles, the oocyte, the wing and the differentiated sense organ (reviewed in Artavanis-Tsakonas et al., 1995). The neurogenic genes *Notch* (*N*), *Delta* (*Dl*), *Suppressor of Hairless* (*Su(H)*) and *Enhancer of split* (*E(spl)*) have been shown to interact genetically, and are thought to function in a common pathway (de la Concha et al., 1988; Vässin et al., 1985;
Verheyen et al., 1996), mastermind (mam) and neuralized (neu) mutations interact with some alleles of N and E(spl), but it is unclear how they fit into the N/Dl pathway (de la Concha et al., 1988; Knust et al., 1987; Xu et al., 1990).

Many of the neurogenic genes encode proteins that have been implicated in signal transduction and cell-fate specification. N and Dl encode transmembrane proteins with EGF-like repeats, and may act as receptor and ligand, respectively (Kidd et al., 1986; Kopczynski et al., 1988; Vässin et al., 1987; Wharton et al., 1985). Su(H) encodes a transcriptional regulator that interacts with the CDC10/ankyrin repeats of N, and is thought to regulate E(spl) expression in response to N signalling (Bailey and Posakony, 1995; Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweiguth, 1995). The E(spl) complex encodes seven bHLH transcriptional regulators and a nuclear protein with a WD-40 motif (Hartley et al., 1988). mam encodes a nuclear protein that may also regulate gene expression (Smoller et al., 1990). neu encodes putative zinc finger and helix-turn-helix motifs, but has no known biochemical function (Boulianne et al., 1991; Price et al., 1993).

As components of the lateral inhibition pathway, each of the neurogenic genes is involved in either generating the lateral inhibition signal or responding to it. This is most evident in the case of N and DI, which are thought to encode receptor and ligand, respectively (Fehon et al., 1990; Heitzler and Simpson, 1991). The genes involved in responding to the signal (e.g. N) act cell autonomously, whereas the genes involved in generating the signal (e.g. DI) act cell non-autonomously.

The big brain (bib) gene is distinct from the other neurogenic genes. Most notably, loss-of-function bib mutations cause less severe neural hyperplasia than loss-of-function mutations in the other neurogenic genes (Lehmann et al., 1983) and bib mutations do not cause defects in sense organ (SO) differentiation (Rao et al., 1992). Furthermore, genetic experiments have failed to reveal genetic interactions between bib and the other neurogenic genes (de la Concha et al., 1988). The N-terminal half of the predicted bib gene product shares sequence similarity with a group of channel proteins that have not been implicated in either cell fate specification or in cell-cell communication, while the C-terminal half does not share homology with any known proteins.

To further elucidate the function of bib, we have tested the ability of the bib cDNA to restore bib function during neurogenesis, examined the tissue-specific expression and subcellular localization of bib protein, determined which cells require bib activity during neurogenesis and found synergistic interactions between ectopically expressed bib and both N and DI. By rescuing the neurogenic phenotype in the embryo, we demonstrate directly that the bib cDNA encodes a functional protein for the signalling events involved in NP specification. Antibody stainings reveal that the bib protein is encoded putative zinc finger and helix-turn-helix motifs, but has no known biochemical function (Boulianne et al., 1991; Price et al., 1993).

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To further elucidate the function of bib, we have tested the ability of the bib cDNA to restore bib function during neurogenesis, examined the tissue-specific expression and subcellular localization of bib protein, determined which cells require bib activity during neurogenesis and found synergistic interactions between ectopically expressed bib and both N and DI. By rescuing the neurogenic phenotype in the embryo, we demonstrate directly that the bib cDNA encodes a functional protein for the signalling events involved in NP specification. Antibody stainings reveal that the bib protein is membrane associated and expressed in tissues that require neurogenic gene activity for their development. Using mosaic analysis, we provide evidence that bib acts cell autonomously, functioning in epidermal cells to help maintain the epidermal fate and inhibit the neural fate. Overexpression of bib with N or DI in the SO lineage causes a cell fate transformation phenotype much stronger than that caused by overexpression of each protein separately. Combined with our observation that bib protein colocalizes with DI and N during neurogenesis, these results suggest that bib is intimately involved in the reception of, or response to, a lateral inhibition signal and is capable of augmenting the activity of the DI-N signalling pathway.

**MATERIALS AND METHODS**

**Drosophila stocks**

All flies were raised at room temperature on cornmeal-agar medium unless otherwise noted. We used the following stocks: ac-lacZ (Van Doren et al., 1991), scgAL4 (Nakao and Campos-Ortega, 1996), hairy-GAL4 (Brand and Perrimon, 1993), GAL410968 (Frisi et al., 1996; Guo et al., 1996) UAS-activated N, UAS-Dl and UAS-N (Doherty et al., 1996). UAS-neu was made by cloning a full-length neu cDNA into the pUAST vector (G. Feger, personal communication). bibFX1, bibC5a, bibD025, E(spl)pD069, DlP19F, neuK92, mam215, Nr55411 and yw; Oregon-R are described in Lindsley and Zimm (1992).

**UAS-bib constructs**

The c13 bib cDNA (Rao et al., 1990), as well as other independently isolated cDNAs, were subcloned into the Xhol and Xbal sites of the pUAS vector (Brand and Perrimon, 1993). These constructs were transformed into yw; Oregon-R flies using standard methods (Laski et al., 1986; Spradling, 1986).

**Immunofluorescence, confocal microscopy and immunoelectron microscopy**

We used the following antibodies: guinea pig anti-myc (G. Feger and Y. N. Jan, unpublished data), guinea pig anti-ase (Rhyu et al., 1994) and monoclonal antibody 22c10 (Zipursky et al., 1984). Rabbit anti-bib was raised against a peptide containing amino acids 672-701 at the bib C-terminus (QQQQQQQQQQQQMQQQQQHYG-MLLPVPN). Antibody staining was competed away by the bib peptide, but not by non-bib peptides (data not shown). Mouse (Kooh et al., 1993) and guinea pig anti-DI were kindly provided by the Muskovitch lab.

We performed immunofluorescence labelings according to Rhyu et al. (1994) with the following modifications. We fixed all samples in PEM5 (0.1 M Pipes pH 6.9, 1 mM EGTA and 2 mM MgSO4) + 4% formaldehyde. Embryos were fixed for 20-30 minutes, while imaginal discs were fixed for 10-20 minutes. All samples were blocked in PBT + 2% normal goat serum. For some of our stainings, we amplified the signal using biotinylated secondary antibodies (Jackson Laboratories, USA), and streptavidin conjugated to DTAF, LRSC or Cy-3. We mounted samples in glycerol/PBS/2% n-propyl-gallate and examined them using a Bio Rad MRC-600 confocal microscope.

We performed immunoelectron microscopy as described in Jongens et al. (1994), using the anti-bib antibody. Clearing embryos for cuticle analysis was performed as described in Ashburner (1989).

**Clonal analysis**

We used the FLP/FRT method developed by Golic and Lindquist (1989) to generate mosaic clones. We constructed chromosomes with a p[ry*; y*]25F (Xu and Rubin, 1993), ck13 (Lindsley and Zimm, 1992), either bibFX1 or bibC5a, and p[ry*; FRT]40A (Xu and Rubin, 1993). To generate the mitotic clones, we mated yw; y* ck bib FRT/CyO males to hsFlp1; p[w*;myc] FRT females (Xu and Rubin, 1993). Flp activity was induced in the progeny at various developmental stages by two shifts to 39°C for 30 minutes separated by 30 minutes at room temperature. Clones were examined in Flp1/; y* ck bib FRT[p[w* myc]] FRT females. Control flies missing Flp1 or either FRT chromosome did not display clones.
RESULTS

bib expression rescues the cuticle and nervous system defects in bib mutant embryos

To determine whether we had generated functional UAS-bib constructs, we drove UAS-bib expression in alternating parasegments using the hairy-GAL4 (h-G4) enhancer trap line IJ3 (Brand and Perrimon, 1993) in bib mutant embryos (Fig. 1I). Embryos mutant for bib develop a hypertrophic nervous system at the expense of the ventral cuticle (Fig. 1C,F and Lehmann et al., 1983); these embryos also have defects in a variety of other tissues (Corbin et al., 1991; Hartenstein et al., 1992 and data not shown). bib- embryos expressing UAS-bib developed significantly more cuticle and displayed less nervous system hypertrophy than their bib- siblings that did not express UAS-bib. A number of rescued embryos developed nearly wild-type thoracic and abdominal cuticle without any missing denticle belts (Fig. 1B), probably because there was low level UAS-bib expression in parasegments that do not express detectable levels of h. Consistent with this hypothesis, UAS-activated N, which acts autonomously, caused some anti-neurogenic effects in all segments when driven by hG4 (data not shown).

As expected, rescue of the peripheral nervous system (PNS) showed some parasegmental periodicity (Fig. 1H). Wild-type embryos almost invariably have 5 lateral chordotonal organs per hemisegment (Fig. 1G), while bib- embryos have an average of at least 10 per hemisegment (data not shown). We observed nearly wild-type numbers of lateral chordotonal organs in T2, A1, A3, A5 and A7 (mean no.=5.1±0.6), but there were extra chordotons in T1, T3, A2, A4, A6 and A8 (mean no.=8.0±1.5). The lateral chordotonal organs arise from the posterior compartment (Hartenstein, 1987), thus the segmental rescue of chordotonal hypertrophy is consistent with the h expression pattern in odd-numbered parasegments. Hypertrophy of the other SOs in the PNS is also decreased in the rescued embryos (Fig. 1G,H). Control crosses in which we left out either UAS-bib or h-G4 did not yield any rescued embryos (data not shown). Finally, rescue was not dependent on a particular UAS-bib insertion site, as we were able to demonstrate rescue using several independent UAS-bib lines. On the basis of these experiments, we conclude that UAS-bib expression can rescue the thoracic and abdominal cuticle defects in bib mutant embryos.

UAS-bib does not rescue the other neurogenic mutants

We tested whether UAS-bib could rescue the phenotype in embryos mutant for other neurogenic genes. UAS-bib expression driven by h-G4 did not rescue the cuticle-defect embryos, Dl, N, E(spl), mam or neu (data not shown). We found that UAS-Dl and UAS-neu did not rescue the neurogenic phenotype in bib mutant embryos. In contrast, UAS-activated N still had an antineurogenic effect in bib mutant embryos, consistent with the results of Lieber et al. (1993). These results indicate that bib, Dl and neu cannot functionally replace each other and that bib functions upstream of or parallel to activated N.

Fig. 1. UAS-bib rescues the cuticle and nervous system phenotypes in bib- embryos. Wild-type (A,D,G), rescued (B,E,H) and bibC7a/bibFX1 neurogenic (C,F) embryos. (A-C) Rescued embryos develop ventral denticle belts (bracket in B), which are completely absent in their unrescued neurogenic siblings (bracket in C). Note that the head cuticle is defective and that the mouth hooks (arrowheads in A) fail to develop in both rescued and neurogenic embryos. (D-F) Monoclonal antibody 22c10 staining for neurons reveals that rescued embryos (E) develop fewer neurons than their unrescued neurogenic siblings (F), but more than their wild-type siblings (D). (G-H) The number of chordotonal neurons in alternate segments of rescued embryos (arrows in H) is nearly the same as in wild-type embryos (arrows in G). (I) bib expression in a stage 9 bibC7a/bibFX1; h-G4 /UAS-bib9-3 embryo. bib protein is membrane-associated and expressed in every other parasegment.
Subcellular localization of big brain protein

We raised a polyclonal rabbit antibody to a peptide encompassing amino acids 672-701 of the translated bib cDNA sequence (see Materials and Methods). Staining with this antiserum was specific for bib protein, as the signal was absent from embryos homozygous for the bib alleles FX1, C7a and II9-5. Embryos mutant for one strongly neurogenic bib allele, ID05, still expressed protein in the mesoderm (data not shown).

We observed bib protein in all plasma membranes during cellularization of the blastoderm embryo (data not shown). The pole cell membranes contained a small amount of bib, which disappeared before gastrulation (data not shown). After cellularization, bib was present in the membranes of all cells and concentrated in a ring around each cell demarcating the border between the apical and basolateral membranes where the cells were tightly apposed (Fig. 2A,B). In addition to the membrane staining, there was also punctate signal within the cytoplasm that may reflect bib protein within intracellular vesicles. Just before gastrulation, a ventral stripe of cells, corresponding to the prospective mesoderm, showed an increase in punctate signal and a decrease in plasma membrane signal (Fig. 2C).

To examine whether bib was associated with specific structures within cells, we performed immunoelectron microscopy on embryos labeled with bib antibody and a secondary antibody conjugated to 1.4 nm gold particles (see Materials and Methods). Using several fixation conditions, we consistently observed grains associated with the plasma membrane of all cells in the neurogenic ectoderm and clusters of grains within the cytoplasm, but very few grains within the nuclei (Fig. 2D,E). The grains associated with the plasma membrane were predominantly on the cytoplasmic face, consistent with the predicted cytoplasmic location of the C-terminal antibody epitope (Rao et al., 1990). The clusters of grains in the cytoplasm were often associated with small vesicles (data not shown). These clusters may correspond to the punctate signal observed in confocal micrographs (compare Fig. 2D,E to Fig. 2A,B). We also observed large numbers of grains associated with the apical adherens junctions described by Tepass et al. (1994). Fig. 2D,E shows one such junction between two neuroectoderm cells in a stage 9 embryo.

bib and Delta proteins colocalize

In the prospective mesoderm just before gastrulation, bib protein disappeared from the plasma membrane (Fig. 2C) and was present in punctate cytoplasmic structures basal to the nucleus. The Delta protein is expressed in a similar manner (Kooh et al., 1993). To address whether bib and Dl colocalize, we simultaneously labeled embryos with bib and Dl antibodies. The bib and Dl proteins did in fact colocalize in the plasma membrane and in the punctate cytoplasmic structures of prospective mesoderm cells (Fig. 3A-C), although the intensity of the two signals was not always similar.

After gastrulation, bib protein continues to be expressed in all cells of the neurogenic ectoderm. In addition to the membrane staining, we found striking punctate localization in the cytoplasm, which was once again coincident with Delta (Fig. 3D-F). We have also found that bib is colocalized with N (data not shown). To explain the punctate Dl staining, it has been proposed that Dl is transported to and from the plasma membrane in vesicles and also undergoes receptor-mediated endocytosis into adjacent N-expressing cells (Kooh et al., 1993). In contrast, we believe that bib remains within the cells where it is expressed, because we have never observed punctate bib staining in cells that do not have bib in the plasma membrane.

To determine whether punctate bib localization was dependent on functional lateral inhibition, we examined bib and DI expression in bib, Dl and N mutant embryos. We found that punctate bib expression in the prospective mesoderm and neurogenic ectoderm was still present in Dl and N mutant embryos. In addition, punctate Dl expression was still present in bib and N mutant embryos (data not shown). These results indicate that while the bib and Dl proteins localize to punctate cytoplasmic structures, this localization does not require N pathway signalling.

bib expression during neuroblast segregation

To determine where and when bib might act during neurogenesis, we examined bib protein expression during neuroblast (Nb) segregation. bib was localized all along the basolateral
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cell membranes of neuroectoderm cells and, in addition to being concentrated apically in the region of the adherens junctions, it appeared to be concentrated basally, where the cells contacted each other and the nascent Nb (Fig. 4A). Concentration of bib and other proteins may be important for efficient signalling between proneural cells.

In stage 10-11 embryos, bib protein was expressed strongly in the epidermis and mesoderm and at a much lower level in the developing CNS, generating a ‘two-stripe’ pattern similar to that described for DI expression (Fig. 4D-F and Kooh et al., 1993). In stage 9 N mutant embryos, all the neuroectoderm cells become Nbs and bib protein was only present in the mesoderm, confirming that Nbs lose bib expression. Using confocal microscopy, it was difficult to determine exactly when
bib protein disappeared from the Nbs, because nascent Nbs are surrounded by the bib-containing membranes of adjacent epidermal cells (Fig. 4C,G); however, in electron micrographs, there were far fewer grains associated with the membranes of segregating Nbs than with the membranes of adjacent neuroectoderm cells, indicating that bib was lost from the Nb membrane early during Nb segregation (data not shown). Thus it appears that the maintenance of the epidermal fate in the neuroectoderm cells surrounding the delaminating Nb does not require the presence of bib protein in the Nb.

**bib is expressed in tissues that require neurogenic gene function**

bib expression was maintained in the epidermis and mesoderm until stage 12. Consistent with the observed *bib* mutant phenotypes in somatic muscles, peripheral glia, oenocytes, optic lobes, stomatogastric nervous system, salivary glands, Malpighian tubules and dorsal vessel (Bate et al., 1993; Corbin et al., 1991; Hartenstein et al., 1992), we observed bib protein expression in all of these tissues or their precursors (Fig. 5 and data not shown). We also observed bib expression in the anterior and posterior midgut invaginations, ventral midline cells and tracheal pit cells (Fig. 5A,B and data not shown). While the midgut, ventral midline cells and trachea are not defective in *bib* mutant embryos, they are all disrupted by loss of other neurogenic gene function (Hartenstein et al., 1992; Menne and Klambt, 1994). Complementary expression of bib and Dl at the midgut/hindgut boundary (Fig. 5F,G) and in rings of cells where the adult midgut precursors form in the proventriculus (data not shown) suggests that these genes play a role in formation of the adult midgut. Finally, we observed conspicuous bib expression in the adult muscle precursors and in subsets of the larval ventral nerve cord and brain lobes (Fig. 5C-E and data not shown); the significance of expression in these tissues is unclear. The bib antibody also results in strong signal in a subset of hemocytes. This signal must represent a cross-reacting antigen, because the signal was not present in *bib*-embryos, it was not competed away by bib peptide and there was no *bib* RNA expression in these cells (data not shown).

In third instar wing discs, we found bib expression in the proneural clusters for wing margin bristles, SOs along the dorsal radius and SOs of the notum (Fig. 5H-J). bib is expressed in the dorsal vessel (dv) and Malpighian tubules (mt); these tissues are defective in bib mutant embryos (Hartenstein et al., 1992). bib is also expressed in the amnio serosa (as), adult muscle precursors (arrowheads) and posterior midgut (white arrow); phenotypes have not been described in these tissues. (G) Dorsal view of a stage 15 embryo. bib (green) is expressed at the posterior limit of the midgut (white arrow) adjacent to a domain of Dl (red) expression at the anterior limit of the hindgut (hg). Dl is expressed in the trachea (tr). (H-J) Third instar ac-*lacZ* wing discs labeled with anti-bib (green in H) and anti-β-galactosidase (red in I), which is expressed in proneural cluster cells. bib is expressed at a high level in proneural clusters (white arrowheads point to scutellar and wing margin clusters), cells at the dorsal/ventral compartment boundary (white arrow), prospective wing veins (labeled 3 and 4), and in adephitelial cells that become the adult flight muscles (bracket). (J) An overlay of H and I.

**Fig. 5.** bib protein is expressed in tissues that are defective in neurogenic mutant embryos. (A) At stage 7, bib is expressed throughout the ectoderm (ec) and in the anterior (a) and posterior (p) midgut invaginations. Many cell types that derive from these regions are defective in neurogenic mutant embryos (Hartenstein et al., 1992). (B) At stage 11 bib is still expressed at a high level throughout the epidermis and in the ventral midline cells (yellow arrow), but not in the developing CNS (bracket). (C) Ventral view of a stage 16 embryo. bib is expressed in a segmentally repeated subset of ventral nervous system cells (yellow arrowheads). (D-F) Lateral views of a stage 13 embryo at two different focal planes. bib is expressed in the dorsal vessel (dv) and Malpighian tubules (mt); these tissues are defective in bib mutant embryos (Hartenstein et al., 1992). bib is also expressed in the amnio serosa (as), adult muscle precursors (arrowheads) and posterior midgut (white arrow); phenotypes have not been described in these tissues. (G) Dorsal view of a stage 15 embryo. bib (green) is expressed at the posterior limit of the midgut (white arrow) adjacent to a domain of Dl (red) expression at the anterior limit of the hindgut (hg). Dl is expressed in the trachea (tr). (H-J) Third instar ac-*lacZ* wing discs labeled with anti-bib (green in H) and anti-β-galactosidase (red in I), which is expressed in proneural cluster cells. bib is expressed at a high level in proneural clusters (white arrowheads point to scutellar and wing margin clusters), cells at the dorsal/ventral compartment boundary (white arrow), prospective wing veins (labeled 3 and 4), and in adephitelial cells that become the adult flight muscles (bracket). (J) An overlay of H and I.
In late third instar wing discs, bib is also expressed in 3-6 rows of cells at the dorsal/ventral boundary of the wing pouch and in the cells that form the wing veins. Despite the fact that N and DI are required for wing margin and wing vein formation, loss of bib function does not disrupt these tissues (data not shown). Finally, bib is expressed at a high level in the cells that later form the flight muscle.

Although bib is expressed in the proneural clusters for macrochaete (Mc) and microchaete (mc) and remains on in epidermal cells, it is off in SOPs (data not shown), and the pIIa and pIIb cells (Fig. 6A-C). In pupae 24 hours after puparium formation (APF), bib is expressed at high levels in clusters of cells around many of the developing Mcs which are composed of 4-cells by this stage (Fig. 6D-F). Where it is not obscured by high level expression in surrounding cells, bib is expressed in all four cells of the Mc and at a high level in the outer cells (Fig. 6G-I), and is still expressed at a low level throughout the epidermis. bib is often most concentrated in the Mc socket cell and in the membranes between the socket and hair cell nuclei. N and Su(H) are also expressed predominantly in socket cells at this stage (Gho et al., 1996), while DI is expressed predominantly in the hair cells (Parks et al., 1997).

In summary, we have found that bib protein expression in the embryo is consistent with the RNA distribution described by Rao et al. (1990). bib is expressed in essentially all tissues where neurogenic gene activity is required. While bib- embryos do not display a phenotype in all these tissues, the expression pattern suggests that bib functions in concert with the other neurogenic genes, or is at least involved in many of the same processes.

**bib functions cell autonomously to inhibit neural development**

To understand how a gene functions in a signalling pathway, it is essential to determine whether the gene is involved in producing the signal or responding to the signal. Proneural cluster cells compete with each other to become NP, so it is possible to determine whether a gene is required to produce or to respond to the lateral inhibition signal by juxtaposing wild-type and mutant cells and examining the resulting cell fates. If bib were involved in producing the signal, the mutant cells would be able to receive the lateral inhibition signal from the adjacent wild-type cells and the mutant cells would develop as epidermis. If bib were involved in responding to the signal, the mutant cells would be unable to receive the lateral inhibition signal from the adjacent wild-type cells and they would develop as supernumerary neural cells.

Using the FLP/FRT system (Golic and Lindquist, 1989; Xu and Rubin, 1993), we generated homozygous bib mutant patches in bib+/bib- adults. We marked the patches with the recessive cuticle marker crinkled (Lindsley and Zimm, 1992). The crinkled (ck) mutation acts cell autonomously, marking all the cells that form the adult cuticle and making it possible to determine the genotype of each cell at clone borders. ck Mc and mc form shortened and thickened hairs, while ck epidermal cells form multiple instead of single epidermal hairs (Fig. 7A,B).

bib mutant clones showed moderately increased numbers of Mc and mc (Rao et al., 1992; Fig. 7A,B; Table 1). The bib- Mc and mc had normal sockets innervated by single neurons, indicating that extra bristles were not generated by cell fate transformation within the sense organ precursor (SOP) lineage (data not shown). Extra bristles were not likely due to extra divisions of the SOPs, because Rao et al. (1992) showed that the extra SOs in bib mutant embryos are not due to extra divisions. In striking contrast to bib- clones, clones mutant for N and DI show a greater degree of bristle hypertrophy and strong alleles of N yield naked cuticle, presumably because all the daughters of the SOPs are transformed into neurons (Heitzler and Simpson, 1991).

At the bib- clone borders, we found supernumerary ck Mc adjacent to ck+ epidermal cells (Fig. 7C). Supernumerary Mc were always ck; therefore when bib+/bib- cells were juxtaposed to bib+/bib- cells in the proneural clusters for Mc, the bib- cells...
often formed supernumerary SOPs. Thus bib cells cannot always be prevented from forming supernumerary SOPs by the adjacent bib+/bib− cells, demonstrating that bib is required to respond to an inhibitory signal.

To determine whether bib− cells can influence their heterozygous neighbors, we counted the numbers of wild-type and ck mc at the clone borders (Table 1). A mc was scored as being on the clone border when it was adjacent to both ck and ck+ epidermal hairs. When bib+/bib− cells and bib+/bib− cells developed at clone borders, bib+/bib− cells produced sense organs three times as frequently as bib+/bib− cells, i.e. there were three times as many ck mc as ck+ mc at the clone borders. The predominance of bib− mc at the clone borders was more than could be accounted for by the very mildly increased mc density within the clones (Table 1). Furthermore, at the borders of control clones marked with ck, we observed ck and ck+ mc with equal frequency. These results indicate that bib− cells have increased signalling capability, similar to N− and E(spl)− cells (Heitzler et al., 1996; Heitzler and Simpson, 1991).

To confirm the cell autonomous action of bib, we examined bib− clones in late third instar wing discs. Using a myc-marked FRT chromosome (Xu and Rubin, 1993), we were able to identify bib− clones by the absence of myc staining (Fig. 7E). Supernumerary SOPs arose adjacent to the clone borders, confirming that formation of the ectopic bib− SOPs was not inhibited by the adjacent wild-type SOPs (Fig. 7F). Our mosaic results in the adult cuticle and third instar wing disc indicate that bib is required for the full reception of or response to an inhibitory signal, and that it is not required for production of the signal.

**Table 1. Microchaete density is increased in bib− clones and bib− microchaete predominate at clone borders**

<table>
<thead>
<tr>
<th>FRT chromosome</th>
<th>Epidermal hairs between microchaete within clones mean ± s.d. (hairs counted)</th>
<th>ck microchaete adjacent to ck+ hairs (microchaete counted)</th>
<th>ck+ microchaete adjacent to ck hairs (microchaete counted)</th>
</tr>
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<tbody>
<tr>
<td>bibFX1 ck</td>
<td>3.49±0.81 (118)</td>
<td>74% (95)</td>
<td>26% (34)</td>
</tr>
<tr>
<td>bibD05 ck</td>
<td>3.66±1.01 (148)</td>
<td>72% (59)</td>
<td>28% (23)</td>
</tr>
<tr>
<td>bib+ ck</td>
<td>4.64±0.98 (166)</td>
<td>52% (96)</td>
<td>48% (88)</td>
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**Overexpression of bib with N or Dl causes synergistic phenotypes**

de la Concha et al. (1988) proposed an epistatic pathway for the neurogenic genes based on whether increasing the wild-type copy number of one neurogenic gene could enhance or suppress the homozygous embryonic phenotype of other neurogenic mutants. Similarly, Vässin et al. (1985) had looked at changes in the haploinsufficient phenotypes of N, DI, E(spl) and H in trans to other mutations. Verheyen et al. (1996) looked for dominant enhancers and suppressors of the activated N overexpression phenotype in the eye. These and other studies established a close genetic relationship between many of the neurogenic genes, but no studies to date have revealed this type of relationship between bib and any other gene. However, these approaches have relied on loss or gain of one copy of a gene resulting in a significant change in the level of activity of the gene product.

We decided to use ectopic expression of bib in vivo to look for genetic interactions that might have been missed using previous methods. Unlike changes in gene copy number, this
type of approach guarantees at least increased transcription of the genes in question and presumably generates significant increases in the resulting gene product. We expressed UAS-

bib in the adult SO lineage using scaGal4 (scaGal4) and GAL4109-68. scaG4 is expressed in proneural cluster cells, SOPs and their daughters (Nakao and Campos-Ortega, 1996). GAL4109-68 is expressed in subsets of proneural cluster cells, SOPs and their daughters (Frise et al., 1996; Guo et al., 1996) and generally gives weaker phenotypes than scaG4. In combination with either GAL4 line, UAS-bib caused mild increases in SOs and rare hair-to-socket transformations in both Mc and mc (Fig. 8B,F). The transformation phenotype is reminiscent of the phenotype caused by N overexpression (Frise et al., 1996; Guo et al., 1996; Lieber et al., 1993; Lyman and Yedvobnick, 1995; Rebay et al., 1993 and Fig. 8D), but it was much less frequent. Overexpression using a weakly expressed UAS-Dl (30A1) caused essentially no

phenotype, while more strongly expressed lines (17c and 30B) caused a moderate increase in SOs (Fig. 8G,I) and extremely rare hair-to-socket transformations (data not shown). It is surprising that bib overexpression caused an increase in the number of SOs, a phenotype similar to that caused by loss of bib function. The same observation is true for DI overexpression and loss of DI function; both cause an increase in the number of SOs. This may be an indication that DI can autonomously inhibit N signal reception in DI-expressing cells (Doherty et al., 1996); when all cells of the proneural cluster express high levels of DI, N signal reception would be inhibited, causing an increase in the number of SOPs. Alternatively, lateral inhibition may require that cells expressing high levels of DI be juxtaposed to cells expressing low levels of DI; both loss of DI function and uniform overexpression of DI would diminish the differences in DI expression levels between cells. Since bib is able to augment DI function when
overexpressed during SO differentiation (see below), bib could also increase DI activity during SOP formation, resulting in slightly increased numbers of SOs.

The cell fate transformation phenotypes caused by coexpression of bib and DI far exceeded the additive combination of the phenotypes caused by expression of the proteins singly. We tried combinations of different UAS lines for each gene with sca/G4 and GAL41098, sca/G4/UAS-bib8-1 flies had fewer than 1 affected notal Mc and 5-10 affected abdominal SOs per fly (Fig. 8B), while sca/G4/UAS-Dl30A flies never displayed transformed SOs (Fig. 8A). The combination, sca/G4/UAS-bib8-1/UAS-Dl30A, caused very frequent hair-to-socket transformations in Mc and Mc on the notum and abdomen (Fig. 8C). In addition, these flies displayed a neuron-to-sheath transformation in pupal nota 24 hours after pupation (data not shown). When driven by sca/G4, UAS-Dl30B and UAS-Dl17c caused a rough eye, slight wing outgrowth, increased numbers of Mc at their normal positions and 3-5 SOs with hair-to-socket transformations on the abdomen of each fly (Fig. 8G and data not shown). The combination of UAS-Dl17c and UAS-bib8-1, driven by sca/G4 or GAL41098, was lethal before pupation, while UAS-Dl30B with UAS-bib8-5 caused a very frequent hair-to-socket transformation phenotype (data not shown).

The interactions between bib and N were less visually striking because the N phenotype alone was quite strong; however, it is clear that bib augments the antineurogenic effect of overexpression of N, as well as cell fate transformation effects (Fig. 8D-F). sca/G4/UAS-N22/UAS-bib8-5 flies had fewer SOs than sca/G4/UAS-N22 flies and the SOs they did have displayed a higher proportion of hair-to-socket cell fate transformations. As a control, we coexpressed N and DI, reasoning that since they have been shown to physically contact each other, they should interact strongly in this type of assay. Indeed, coexpression of UAS-N22 and UAS-Dl17c, with either sca/G4 or GAL41098 caused an enhancement of the UAS-N22 phenotype (Fig. 8G-I and data not shown). This same UAS-Dl line (17c) in combination with UAS-bib8-1 caused lethality when driven by either sca/G4 or GAL41098. In addition, UAS-Dl30A, which strongly enhanced the sca/G4/UAS-bib8-1 phenotype, had little effect on the sca/G4/UAS-N22 phenotype. Thus, the interactions between UAS-bib and UAS-Dl and between UAS-bib and UAS-N are at least as strong as the interaction between UAS-N and UAS-Dl.

We have found that bib strongly enhances the N overexpression phenotype during SOP selection, a time when both bib and N are known to function. Although bib mutants do not display a SO cell fate transformation phenotype, we have found that bib can enhance the cell fate transformation phenotype caused by N or DI overexpression. We doubt that the enhancement of DI-N signalling is due to a non-specific effect of overexpressing a transmembrane or adherens junction protein. This type of non-specific effect would more likely disrupt DI-N signalling. Using the rescue assay described above, it will be possible to define domains of the bib protein required for membrane localization, concentration at adherens junctions, rescue of the bib phenotype and synergism with DI and N. It will be interesting to see whether mutant bib proteins that are localized normally but do not rescue the bib phenotype still activate DI-N signalling. These experiments will provide the best controls to see whether overexpression bib protein non-specifically activates DI-N signalling.

The strong nature of the bib-DI and bib-N interactions provides the first evidence that bib is at least capable of acting in concert with N and DI.

**DISCUSSION**

**bib cDNAs rescue the bib phenotype**

The bib cDNA sequence predicts a gene product with similarity to the MIP family of small molecule channel proteins (Rao et al., 1990). This channel family includes many proteins, from the CHIP28 water channel in humans to the glycerol facilitator protein in *E. coli* (reviewed in Chrispeels and Agre, 1994). Many of the proteins have been shown to allow transport of water and small molecules like glycerol across membranes, but they have not been implicated in cell-cell communication or cell fate decisions. We have provided direct confirmation that the bib gene product is indeed involved in the neural versus epidermal cell fate choice. In addition, rescue using h-G4-driven bib expression provides an excellent assay for in vivo bib gene activity.

**bib encodes a membrane-associated protein that colocalizes with the Notch and Delta proteins**

In all cells where bib protein is expressed, it is associated with the plasma membrane and punctate cytoplasmic structures. The punctate staining may represent bib protein in the endoplasmic reticulum, in the Golgi or in vesicles on their way to and/or from the plasma membrane. As with DI antibody staining, there is a striking increase in punctate staining in the cells of the prospective mesoderm, concomitant with loss of membrane staining. Koo et al. (1993) have proposed that the increased vesicular staining represents down-regulation of DI protein from the surface of these cells. We believe that bib is down-regulated in a similar manner.

A number of proteins involved in signalling are internalized and transported to multivesicular bodies. In *Drosophila*, these proteins include the receptor tyrosine kinase product of the *sevenless* gene, its ligand, the *bride of sevenless* gene product and the secreted product of the *wingless* gene (Cagan et al., 1992; Gonzalez et al., 1991; van der Heuvel et al., 1989). With fixation conditions that gave strong plasma membrane signal, we did not find grains associated with multivesicular bodies in any of the cells of the neuroectoderm (data not shown). The absence of signal probably indicates the absence of bib protein, although we cannot rule out inadequate penetration of the antibody, masking of the peptide epitope or degradation of the protein in multivesicular bodies.

Although bib and the other neurogenic genes share many of the same loss-of-function phenotypes, bib has not been shown to interact genetically with these genes. Even so, bib, DI and N display similar patterns of subcellular localization; therefore bib must function in physical proximity to these proteins and might even contact DI and N directly or via other proteins. Physical interaction between bib and N is suggested by strong enrichment for bib sequences in experiments wherein a library of *Drosophila* embryonic cDNA-derived peptides, displayed on the surface of filamentous phages, was screened on the surface of S2 cells expressing N (C. Wesley, A. S. Manoukian and M. Young, personal communication). It will be interesting to test for physical association between bib, N, DI and the other neurogenic gene products using the yeast interaction trap
system (Chien et al., 1991), interaction assays in cultured cells (Fortini and Artavanis-Tsakonas, 1994) and by more conventional biochemical methods.

**bib protein is expressed in tissues affected by neurogenic mutations**

We have shown that *bib* protein is expressed in a pattern consistent with the previously described pattern for the *bib* transcript (Rao et al., 1990). It is expressed in all the cells of the neurogenic ectoderm before Nb segregation, consistent with its proposed role in lateral inhibition. The fact that bib expression turns off in the Nbs prior to division suggests that *bib* activity may be required only in the epidermal cells. Alternatively, bib may be required in all the cells, but only during the early events of Nb selection before segregation.

The bib expression pattern suggests that bib functions in concert with the other neurogenic genes. bib is expressed during the development of virtually all tissues defective in neurogenic mutant embryos, even in tissues such as the trachea that are not defective in *bib* mutant embryos. The absence of a *bib* mutant phenotype in tissues where it is expressed could be due to a variety of reasons. (1) We have not examined these tissues carefully enough to discern a subtle *bib* mutant phenotype. (2) In some tissues, loss of *bib* function could be compensated for by a gene or genes with similar or dissimilar gene products. (3) *bib* could function via a tissue-specific component similar to Ser or dx (Diederich et al., 1994; Speicher et al., 1994). (4) The expression could be an evolutionary relic in tissues that have lost their requirement for *bib*.

**bib is required in epidermal precursors to respond to the lateral inhibition signal**

Our mosaic studies in adults provide strong evidence that *bib* is required autonomously in the epidermal precursors. In *bib* clones, supernumerary bristles are formed at the clone borders, immediately adjacent to wild-type cells. This suggests that when a *bib* cell develops adjacent to wild-type cells in a proneural cluster, it cannot consistently respond to the wild-type inhibitory signal and is therefore more likely to become a NP. Thus *bib* function is required for the proper reception of or response to the lateral inhibition signal.

While *N* and *bib* both act autonomously, there are differences in their mosaic phenotypes. The increase in bristle density in *bib* mutant clones is significantly milder than in *N* mutant clones (Heitzler and Simpson, 1991; Rao et al., 1992). This is consistent with the milder *bib* neurogenic phenotype in the embryo (Lehmann et al., 1983; Rao et al., 1992). A second difference is that bristles fail to form in clones of cells mutant for null *N* alleles where all four SO cells are presumably transformed into neurons (Hartenstein and Posakony, 1990). Clones of cells mutant for null *bib* alleles show no bristle differentiation defects, consistent with the absence of PNS differentiation defects in *bib* mutant embryos. Finally, Heitzler and Simpson (1991) have shown that cells with lower *N* activity prevent adjacent cells with higher *N* activity from becoming neural. Complete loss of *bib* activity only modestly increases the ability of cells to inhibit their neighbors. These data further confirm that *bib* cannot be a universally required downstream effector of the *N* pathway, despite the fact that it is required in the signal receiving cells during neurogenesis.

**Models for bib function**

We have provided evidence that *bib* is a membrane-associated protein required for the accurate reception of or response to the lateral inhibition signal, and it is expressed in the right locations to function in concert with the other neurogenic genes. We have also found that bib levels adequate to rescue the loss-of-function *bib* phenotype are not adequate to rescue the loss-of-function phenotypes of the other neurogenic genes. Further, *N* pathway activity cannot depend strictly on *bib* function, because the loss-of-function *bib* phenotype is only a subset of the loss-of-function *N* phenotype, and because the phenotype caused by expression of activated forms of *N* is not suppressed by loss of *bib* function (Lieber et al., 1993). These data constrain the mechanisms by which *bib* could act.

It has been proposed that *bib* functions in a pathway parallel to the *N* pathway, because *bib* has not been shown to interact with the other neurogenic genes (de la Concha et al., 1988). *bib* could be involved in the reception of a second epidermalizing signal from the NP to the surrounding cells or in the reception of a signal between the proneural cluster cells that become epidermal. *bib* could play an analogous role in other tissues. While it remains possible that *bib* functions in parallel to the *N* pathway, we feel that the *bib* phenotype, protein distribution and synergistic overexpression phenotypes provide suggestive evidence that *bib* functions at least in part together with the other neurogenic genes. Recently, Seugnet et al. (1997) have proposed a mechanism for NP segregation where DI-N signalling plays a role at two different steps. In the first step, DI-N signalling would inhibit all cells of the proneural cluster, preventing all but the few with the highest neural potential from becoming neural. Feedback regulation of *N* and DI expression would not be required for this step. In the second step, DI-N signalling would provide direct feedback between the few cells with the highest neurogenic potential, ensuring that only one NP is formed. It could be that *bib* is required only for this second phase of DI-N signalling, explaining why loss of *bib* function results in only a 2- to 3-fold increase in SOs.

We favor a model where bib would act in the signal-receiving cell to potentiate N/DI binding or to potentiate the signal generated by *N* in response to DI binding. Loss of *bib* function would cause a phenotype only when very efficient signalling is required, such as during rapid neuroblast segregation in the embryonic CNS. When there is more time and fewer cells involved, such as during differentiation of SOs, the phenotype would be negligible. It will be possible to further elucidate the mechanism of *bib* function by defining the protein domains required for activity and screening for proteins that interact with bib.

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REFERENCES


numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell 76, 477-491.


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