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Genetic separation of the neural and cuticular patterning functions of *gooseberry*

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SUMMARY

In addition to their role in the specification of the epidermal pattern in each segment, several segment polarity genes, including *gooseberry (gsb)*, specify cell fate in the *Drosophila* central nervous system (CNS). Analyses of the *gsb* CNS phenotype have been complicated by the fact that the previously available *gsb* mutants, all caused by cytologically visible deficiencies, have severe segmentation defects and also lack a number of additional genes. We have characterized two novel *gsb* mutants which, due to their hypomorphic nature, have CNS defects, but have only weak or no segmentation defects. These *gsb* alleles, as well as *gsb* rescue experiments, have allowed us to determine which aspects of the deficiency mutant phenotypes can be attributed to loss of *gsb*. *gsb* mutants lack U and CQ neurons, have duplicated RP2 neurons, and display posterior commissure defects. *gsb* neural defects, as well as the *gsb* cuticle defect, are differentially sensitive to the level of functional Gsb. We have used one of the novel *gsb* alleles in order to understand the genetic interactions between *gsb*, *wingless* (*wg*), and *patched* (*ptc*) during the patterning of the ventral neuroectoderm. In contrast to epidermal patterning, where Gsb is required to maintain *wg* transcription, we find that Gsb antagonizes the Wg signal that confers neuroblast (NB) 4-2 fate.

Key words: *gooseberry*, *wingless*, *patched*, neurogenesis, neuroblast, *Pax*, *Drosophila*

INTRODUCTION

A number of *Drosophila* segmentation genes are responsible for generating both epidermal and neural pattern within each segment. The segment polarity gene *gooseberry* (*gsb*), which encodes a transcription factor, has been suggested to have such a dual function. Deletion of the *gsb* locus causes both alteration in the epidermal pattern (Nüsslein-Volhard and Wieschaus, 1980) and pattern defects in the underlying nervous system (Patel et al., 1989). Unfortunately, trying to separate the neural patterning functions of a segmentation gene from its epidermal patterning functions can be quite difficult. In the case of *gsb*, such an analysis is further complicated by the complex molecular nature of the *gsb* locus.

The *gsb* locus was initially analyzed using two deficiencies, *Df(2R)IIX62* and *Df(2R)Kr^{SB1}* (Nüsslein-Volhard et al., 1984). Later, it was discovered that the *gsb* locus actually contains two adjacent transcription units (Bopp et al., 1986; Baumgartner et al., 1987): *gooseberry* (*gsb*; also known as *gooseberry-distal*, *gsb-d*, Côté et al., 1987) and *gooseberry neuro* (*gsbn;* also known as *gooseberry-proximal*, *gsb-p*, Côté et al., 1987). *gsb* and *gsbn* share extensive homology with each other and with the pair-rule gene *paired* (*prd*). The homologous regions include the paired-domain and the *prd*-type homeodomain (Bopp et al., 1986).

The *gsb* locus and the extent of two deficiencies in the region are depicted in Fig. 1A. Even the *trans*-heterozygous combination between *Df(2R)IIX62* and *Df(2R)Kr^{SB1}* deletes a number of genes in addition to *gsb*. Mutagenesis screens attempting to identify segmentation mutants (Nüsslein-Volhard et al., 1984) or embryonic lethal mutations which do not complement *Df(2R)IIX62* (Côté et al., 1987) failed to produce point mutations in *gsb* or *gsbn*, forcing researchers to try to work with the two *gsb* deficiency alleles in order to analyze the functions of *gsb* and *gsbn* in epidermal and CNS patterning (Nüsslein-Volhard and Wieschaus, 1980; Côté et al., 1987; Patel et al., 1989; Li and Noll, 1993; Gutjahr et al., 1993; Zhang et al., 1994; Skeath et al., 1995; Bhat, 1996).

gsb deficiency mutants have cuticle defects (resulting from mis-patterning of the epidermis) consisting of mirror image duplications of denticle belts into regions which would normally contain naked cuticle (Nüsslein-Volhard and Wieschaus, 1980). Gutjahr et al. (1993) demonstrated that *gsb*, in the absence of *gsbn*, could completely rescue the cuticular segmentation defects found in *gsb* deficiency mutants, thus demonstrating that the deficiency epidermal phenotype could

be attributed solely to a loss of *gsb*. During epidermal patterning, Gsb plays a role in maintaining *wg* expression through a *wg-gsb* autoregulatory loop (Li and Noll, 1993). The *gsb* cuticle phenotype, which resembles that of a late wg^{ts} temperature shift, is due to this late loss of *wg* expression in the epidermis and is less extreme than the complete loss of naked cuticle observed in *wg* null mutants (reviewed by Peifer and Bejsovec, 1992).

Patel et al. (1989) demonstrated that in addition to its role in cuticle patterning, *gsb* functions in *Drosophila* CNS development. Unfortunately, the *gsb* epidermal defect made analysis of the *gsb* CNS defects extremely difficult. Furthermore, since the deficiency alleles used in this and other studies remove genes with neural transcripts, it was impossible to be certain which, if any, aspects of the deficiency phenotypes could be attributed solely to a loss of *gsb*.

We have identified two mutations in *gsb* which allow us to analyze the role of *gsb* in CNS development in more detail. Our studies have been aided by the fact that the mutants, genetic hypomorphs, have weak or no epidermal phenotypes, making analysis of their CNS defects much more straightforward. The new mutant alleles, as well as *gsb* rescue experiments, have enabled us to determine which aspects of the *gsb* deficiency mutant CNS phenotypes are due to loss of *gsb*. Analysis of the *gsb* mutant alleles has allowed us to create an allelic series of *gsb* mutants. Interestingly, our results indicate that the CNS and epidermal phenotypes of *gsb* mutants are differentially sensitive to the dosage of functional Gsb protein.

Work with one of the *gsb* alleles has also enabled us to study the interactions among segment polarity genes during the patterning of the neuroectoderm. We define the neuroectoderm to be the monolayer of cells that gives rise to both NBs and epidermoblasts during stages 8 through 11. We define the epidermis to be the monolayer of cells containing epidermoblasts that remains (after the NBs have delaminated) from stage 11 onward. Previous work showed that Gsb positively regulates *wg* transcription during epidermal patterning (Li and Noll, 1993). Our analysis, as well as the work of Bhat (1996), has uncovered novel genetic interactions between *gsb* and *wg* which occur earlier in development, during the patterning of the neuroectoderm. In the neuroectoderm, Gsb antagonizes at least one aspect of Wg signaling. The Wg signal, secreted by row 5 neuroectodermal cells, confers the NB 4-2 fate on cells lying just anterior to the *wg* expression domain (Chu-LaGraff and Doe, 1993). In row 5, Gsb antagonizes the NB 4-2 specifying function of Wg, thus ensuring that none of the Wg-expressing cells take on the NB 4-2 fate. Interestingly, Wg also acts to maintain *gsb* expression during this time period (Li and Noll, 1993; this work). Thus, a secreted molecule confers a particular cell fate; at the same time, the signaling molecule regulates the expression of a transcription factor within the cells that secrete the signal, preventing these cells from taking on the fate conferred by the signal. Finally, the transcription factor also acts later to maintain the expression of the signaling molecule. This scenario provides a scheme in which the coupling of a signaling molecule and a transcription factor can act to both limit the response of cells to the signal and subsequently maintain the signal. This may represent a theme common to many signal transduction and cell patterning systems.

MATERIALS AND METHODS

Fly strains

The following stocks, described by Lindsley and Zimm (1992), were used in this study: *Df(2R)IIX62*, $Df(2R)Kr^{SB1}$, wg^{CX4} (null), wg^{IL114} (temperature-sensitive), and ptc^{IN108} (strong). Enhancer trap *Y72*, obtained from C. Goodman, labels the pCC neurons on the dorsal surface of the CNS. Additional neurons in the CNS are also labeled in *Y72*, but these cells appear later in development and are more ventrally located.

gsb^{525} was recovered in the Seeger et al. (1993) screen and was initially mapped to the *gsb* region based on its inability to complement *Df(2R)IIX62*. Rescue of gsb^{525} by a *gsb* rescue construct (see below) indicated that this was a mutation in the *gsb* gene. Sequencing demonstrated that Gln185 (CAA) of *gsb* (Baumgartner et al., 1987) is converted to a UAA stop codon, the preferred stop codon in flies (see below; Fig. 1C). Although we detect *gsb* transcript in gsb^{525} mutants by in situ hybridization, Gsb protein could not be detected with Gsb polyclonal (Gutjahr et al., 1993) or monoclonal antibodies (Zhang et al., 1994). Our inability to detect Gsb protein in these mutants suggests three possible explanations for the gsb^{525} phenotype: (1) gsb^{525} mutants make a partially functional truncated Gsb protein which cannot be recognized by the Gsb antibodies (if epitopes recognized by the antibodies are C-terminal to the homeodomain); (2) a partially functional, but highly unstable, truncated Gsb protein is made, but cannot be detected due to its instability; or (3) a small amount of full length protein, too little to be detected by Gsb antibodies, is made by reading through the stop codon. Future studies will address the precise molecular nature of this allele, but for the purposes of the work described here, the relevant information is that gsb^{525} acts as a hypomorphic allele of *gsb* (see below).

gsb^{P1155} was discovered in the Spradling P-element collection (Karpen and Spradling, 1992) in a screen for P-lethals affecting Even-skipped (Eve) neural expression (N. Patel, unpublished). Rescue of gsb^{P1155} by a *gsb* rescue construct, as well as reversion by excision of the P-element (see below), indicated that this was a mutation in the *gsb* gene. The gsb^{P1155} phenotype (discussed below) likely results from a decrease in *gsb* transcription due to the P-element insertion into the *gsb* promoter region (Fig. 1B). Preliminary in situ analysis did not reveal an obvious reduction in *gsb* transcript in gsb^{P1155} mutants, but the degree of reduction might not be clearly indicated by whole-mount in situ hybridization. Future studies will address details of the molecular nature of the gsb^{P1155} allele, but as with gsb^{525}, we have utilized its hypomorphic nature in our genetic studies described here.

wg^{CX4} gsb^{525}, wg^{IL114} gsb^{525}, and ptc^{IN108} gsb^{525} stocks were constructed through genetic recombination. At least two independent recombinants for each double mutant combination were generated and studied.

Cloning and sequencing of gsb^{525} and gsb^{P1155} alleles

Genomic DNA isolated from gsb^{525}/*CyO* and gsb^{P1155}/*CyO* flies was analyzed by Southern blot hybridization using various genomic DNA probes covering the *gsb* gene. This analysis revealed no gross DNA deletions or rearrangements in gsb^{525}. Various primer combinations were used to PCR amplify and sequence both strands of the *gsb* coding region from gsb^{525}/*CyO* flies. These sequencing reactions were run alongside parallel reactions done with wild-type genomic DNA. Amplification and sequencing with the primers 5′-GAGTCCA-GAATCGAGGA-3′ and 5′-GCGGCACTGGTAGTGGG-3′ revealed that the CAA codon encoding 185Gln is mutated to a UAA stop codon in gsb^{525} mutants.

For gsb^{P1155}, Southern blot hybridization revealed that the P-element insertion was within a 373 bp *Eco*RI fragment that includes the transcription initiation site for *gsb* (Li et al., 1993). Flanking sequences from the P-element insertion site were recovered by plasmid rescue (starting with genomic DNA from gsb^{P1155}/*CyO* flies that was double digested with *Xba*I and *Nhe*I). The resulting 'rescue plasmid' contained approximately 2.5 kb of genomic DNA flanking

the P-element insertion site. The junction between the P-element and genomic DNA (Fig. 1B) was determined by sequencing the rescue plasmid using primers corresponding to the end of the P-element.

Excision of P-element in *gsb^{P1155}*

88 *ry*$^{-}$ revertants of *gsb^{P1155}* were generated by using the stable Δ2-3 source of transposase. 54 of the revertants were homozygous viable and could complement the original insertion allele as well as *Df(2R)IIX62*. Southern blot analysis of DNA from a random selection of 12 of these viable revertant lines indicated that these were precise excision events. The remaining 34 revertants were homozygous lethal and failed to complement the original insertion as well as *Df(2R)IIX62*. Of these 34 lethal revertants, 16 were terminal deletions of the tip of the 2R chromosome with the breakpoints beginning at various points within the original P-element DNA. The remaining 18 lethal revertants were all internal rearrangements of the initial P-element, and in none of these were any flanking genomic DNA sequences deleted. All 18 of these revertants, when placed *trans* to *Df(2R)IIX62*, could be rescued to adulthood by a single copy of the *gsb* P-element rescue construct (Gutjahr et al., 1993).

Immunostaining and in situ hybridization

Embryo collection, fixation and histochemical staining were carried out as discussed by Patel (1994). Monoclonal antibody BP102 (A. Bieber, N. Patel, and C. Goodman, unpublished) reveals the patterns of the longitudinal and commissural axons in the CNS. The Eve monoclonal antibody (mAb 2B8; Patel et al., 1994), Wg polyclonal antibody (Martinez Arias et al., 1988), and Gsb antibodies (Gutjahr et al., 1993 - polyclonal; Zhang et al., 1994 - monoclonal) have been described. Polyclonal anti-β-galactosidase antibody was obtained from Cappell.

In situ hybridization was completed according to the method of Patel (1996). For all expression studies, mutant chromosomes were balanced over a *CyO, hb-lacZ* balancer so that homozygous mutant embryos could be recognized by their lack of β-galactosidase expression.

gsb rescue experiment

Three independent transgenic lines containing the same previously described *gsb* P-element rescue construct (Gutjahr et al., 1993) were used to create the following genotypes: (1) *gsb^{525}/Df(2R)Kr^{SB1}; P[ry^{+}, gsb^{+}]/+*, (2) *gsb^{525}/Df(2R)IIX62; P[ry^{+}, gsb^{+}]/+*, (3) *gsb^{P1155}/Df(2R)Kr^{SB1}; P[ry^{+}, gsb^{+}]/+*, (4) *gsb^{P1155}/Df(2R)IIX62; P[ry^{+}, gsb^{+}]/+*, (5) *Df(2R)IIX62/Df(2R)Kr^{SB1}; P[ry^{+}, gsb^{+}]/+* and (6) *Df(2R)IIX62/Df(2R)IIX62, P[ry^{+}, gsb^{+}]/+*. In all cases, all three transgenic rescue lines gave similar results. For the first four genotypes (1-4), complete rescue of the CNS defects and embryonic lethality was observed, and a number of animals survived to adulthood. Specific aspects of the rescue of genotypes 5 and 6 are described in the Results section.

RESULTS

Molecular characterization of *gsb^{525}* and *gsb^{P1155}* alleles

gsb^{525} and *gsb^{P1155}* mutant alleles were cloned and sequenced. Sequence analysis indicates that in *gsb^{525}*, CAA encoding 185Gln, the first amino acid of the homeodomain (Fig. 1C), is mutated to a UAA stop codon. Sequencing the *gsb^{P1155}* allele indicates that it has a P-element inserted in the *gsb* promoter region at position –46 relative to the transcription initiation site (Fig. 1B). Additional details concerning these alleles are provided in the Materials and Methods section.

A lack of epidermal defects in the hypomorphic alleles *gsb^{525}* and *gsb^{P1155}*

The *gsb* deficiency cuticle defect, which is completely rescued by the *gsb* transgene (Gutjahr et al., 1993), results from defects in the epidermis and consists of a lack of denticle repression in areas which would normally have naked cuticle (Nüsslein-Volhard and Wieschaus, 1980).

gsb^{P1155} mutants do not have a *gsb* cuticle phenotype, while *gsb^{525}* mutants occasionally have subtle cuticle defects (Table 1). Lack of a cuticular phenotype in *gsb^{P1155}* and *gsb^{525}* mutants correlates with the presence of Wg protein in these mutants during stages 11 through 13. In *Df(2R)IIX62/Df(2R)Kr^{SB1}* mutants (Hidalgo and Ingham, 1990) or *Df(2R)IIX62* homozygotes (Fig. 5E), ventral *wg* expression completely disappears during stage 11. This time period corresponds to the beginning of Gsb-dependent Wg autoregulation (Li and Noll, 1993). In *gsb^{P1155}* mutants, Wg expression appears normal (not shown). In *gsb^{525}* mutants, Wg expression is greatly reduced (Fig. 5F), but detectable during the period of Wg autoregulation. Therefore, we conclude that *gsb^{P1155}* and *gsb^{525}* mutants have

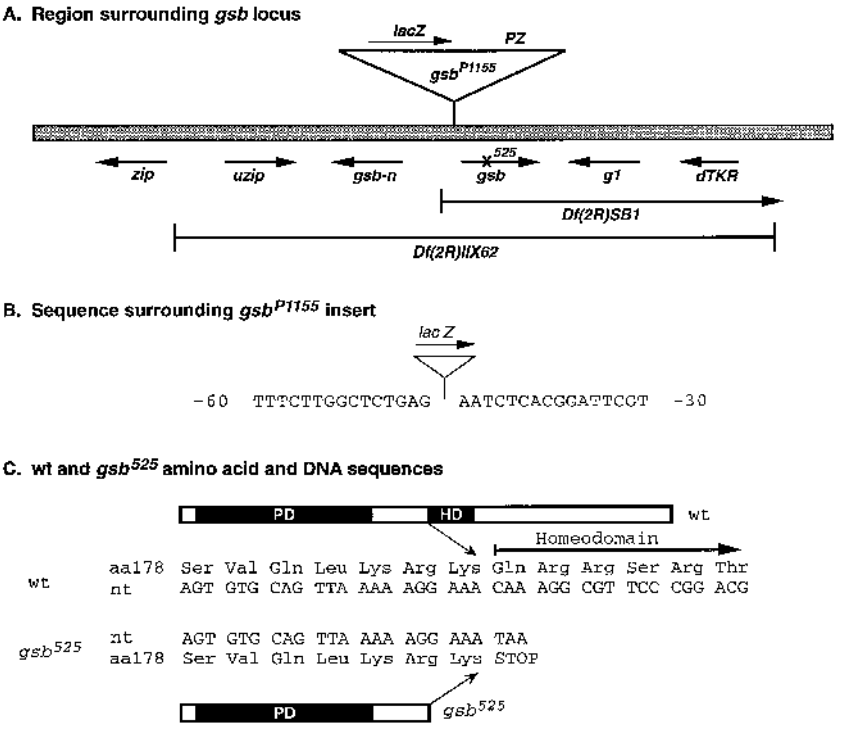

Fig. 1. Region surrounding *gsb* locus. (A) The *gsb* locus and genes which surround it, as well as the extent of the deficiencies *Df(2R)IIX62* and *Df(2R)Kr^{SB1}* are indicated (Côté et al., 1987; Haller et al., 1987; Young et al., 1993). (B) The *gsb^{P1155}* P-element insertion point in the *gsb* promoter region (Li et al., 1993) is depicted. (C) Wild-type (Baumgartner et al., 1987) and *gsb^{525}* DNA and protein sequences are shown. In *gsb^{525}*, the codon for the first amino acid of the homeodomain (HD), 185 Gln, is converted to a stop codon. This may produce a truncated protein with a paired-domain (PD), but no HD.

Table 1. An allelic series of *gsb* CNS and cuticle defects

Genotype	# Duplicated RP2s/hemisegments counted	% RP2 duplication	# Mutant cuticle segments/segments counted	% Segments with cuticle defect
Oregon-R	0/90	0	0/280	0
P1155/P1155	15/72	21±4.8	0/352	0
525/P1155	38/88	43±5.3	ND	ND
P1155/IIX62	33/72	46±5.9	10/400	2.5±0.8
525/525	58/72	81±4.7	12/312	3.8±1.1
525/IIX62	68/90	76±4.6	130/416	31±2.3
IIX62/SB1	49/60	82±5.0	248/256	97±1.1

The percentages of RP2 duplication and mutant cuticle segments and their standard deviations are listed for various *gsb* mutants. Standard deviations were calculated assuming that the development of every hemisegment (for RP2 duplication) or segment (for mutant cuticle) is independent of all others and that the results fit a binomial distribution. The RP2 and cuticle defects are sensitive to the level of functional Gsb protein. As the level of functional Gsb protein decreases, the percentage of RP2 duplication and the penetrance of the cuticle defect increases.

enough Gsb and Wg activity to generate a wild-type cuticle. Although gsb^{P1155} and gsb^{525} mutants have weak or no cuticle defects, cuticle defects appear in gsb^{P1155}/*Df(2R)IIX62* larvae and are considerably enhanced in gsb^{525}/*Df(2R)IIX62* mutants (Table 1), a phenomenon discussed in more detail below.

Analysis of the *gsb* CNS phenotype

Df(2R)IIX62/Df(2R)Kr^{SB1} mutants were previously reported to exhibit duplicated RP2 neurons (Fig. 2B), loss of U and CQ neurons (Fig. 2G), loss or reduction of the posterior commissure, and duplication of aCC and pCC neurons (Patel et al., 1989). However, the epidermal phenotype of the deficiency mutants made it difficult to identify individual neurons with complete certainty. We examined gsb^{525} and gsb^{P1155} mutants in order to gain a better understanding of the *gsb* phenotype.

gsb^{525} mutants have duplicated RP2 neurons (Fig. 2C, Table 1, Fig. 4B) and a loss of many U and CQ neurons (Fig. 2H). These mutants do not appear to have duplicated aCC and pCC neurons. To confirm that the RP2 neurons are duplicated while the aCC and pCC neurons are not, the *Y72* enhancer trap (which labels the pCC neurons) was crossed into the gsb^{525} background. While this experiment confirms that the RP2 neurons are duplicated, we see no indication that the aCC or pCC neurons are duplicated (Fig. 3B). The *Y72* enhancer trap was also crossed into the *Df(2R)IIX62* background. With the enhanced ability to identify cells through the aid of the enhancer trap, we see no indication that aCC and pCC neurons are duplicated in *Df(2R)IIX62* homozygotes (Fig. 3C). The mistake in the initial *Df(2R)IIX62/Df(2R)*Kr^{SB1} characterization (Patel et al., 1989) was probably due to the fusion of adjacent neuromeres in the deficiency mutants.

gsb^{525} mutants have commissural axon defects much like those of *Df(2R)IIX62/Df(2R)*Kr^{SB1} mutants (Patel et al., 1989). The posterior commissure is missing or reduced in each

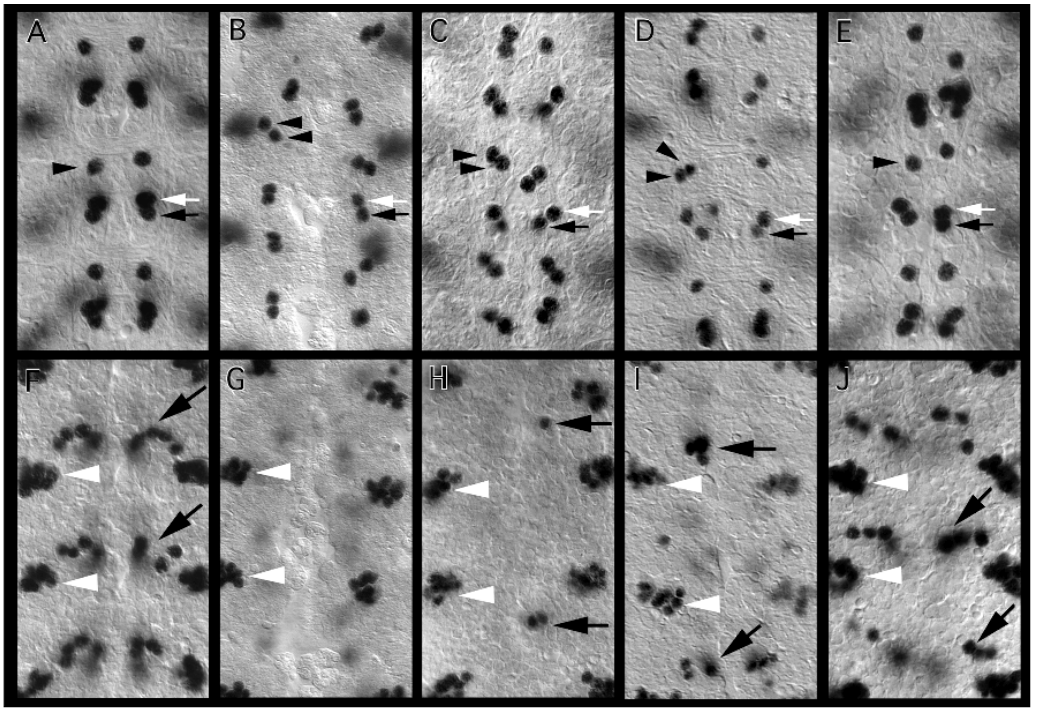

Fig. 2. *gsb* mutants have duplicated RP2 neurons and lack U and CQ neurons. Dorsal (A-E) and ventral (F-J) views of Eve-expressing neurons in stage 15 wild-type (A,F), *Df(2R)IIX62/Df(2R)*Kr^{SB1} (B,G), gsb^{525} (C,H), gsb^{P1155} (D,I), and *Df(2R)IIX62/Df(2R)*Kr^{SB1}; $P[ry^+, gsb^+]$/+ (E,J) embryos are shown. In all panels, anterior is up. Wild-type embryos (A) have single RP2 (black arrowhead), aCC (white arrow), and pCC (small black arrow) neurons in each hemisegment. RP2 neurons are duplicated, but aCC and pCC neurons are unaltered in *gsb* mutants (B-D). Eve-expressing U and CQ neurons (large black arrow) and EL neurons (white arrowhead) are visible in stage 15 wild-type embryos (F). EL neurons are unaltered in *gsb* mutants (G-I). *Df(2R)IIX62/Df(2R)*Kr^{SB1} embryos lack U and CQ neurons (G). gsb^{525} (H) and gsb^{P1155} (I) mutants lack many U and CQ neurons. *Df(2R)IIX62/Df(2R)*Kr^{SB1}; $P[ry^+, gsb^+]$/+ rescued embryos have single RP2 neurons (E) and many restored U and CQ neurons (J).

segment (Fig. 3E). As in *Df(2R)IIX62/Df(2R)Kr^{SB1}* mutants, the commissure phenotype varies from segment to segment, but no segment is completely normal. Although previous studies (Ouellette et al., 1992) had suggested that the *Df(2R)IIX62/Df(2R)Kr^{SB1}* posterior commissural phenotype results from a loss of *Drosophila tyrosine kinase-related* (*dTKR*; Fig. 1A; Haller et al., 1987), analysis of *gsb^{525}* mutants indicates that this commissural phenotype is due to loss of *gsb*.

gsb^{P1155} mutants have a similar but weaker CNS phenotype compared to that of *gsb^{525}* mutants. *gsb^{P1155}* mutants show occasional duplication of RP2 neurons (Fig. 2D, Table 1), frequent loss of U and CQ neurons (Fig. 2I), and reduced or absent posterior commissures (not shown). Although *gsb^{P1155}* is a weaker allele than *gsb^{525}*, its characterization provides further verification for our interpretation of the *gsb* CNS phenotype. Furthermore, *gsb^{P1155}* is particularly interesting in relation to *gsb* dosage studies (see below).

Rescue of the *gsb* CNS phenotype

In order to determine that the mutations in *gsb* are completely responsible for the phenotypes of *gsb^{525}* and *gsb^{P1155}* mutant embryos, we attempted to rescue embryos carrying these alleles in heterozygous combinations over either of the two *gsb* deficiencies with one copy of a *gsb* transgene *P[ry$^+$, gsb$^+$]* (Gutjahr et al., 1993; see genotypes 1-4 in the Materials and Methods section). These rescued embryos show no neural defects in Eve or BP102 staining patterns and have no cuticle defects. All rescued mutants hatched, and a small percentage survived to adulthood. *gsb^{P1155}* mutants could also be rescued by precise excision of the P-element. These results indicate that the mutant phenotypes that we observe result from mutations in *gsb*.

Rescue of neural defects in *Df(2R)IIX62/Df(2R)Kr^{SB1}; P[ry$^+$, gsb$^+$]/+* and *Df(2R)IIX62/Df(2R)IIX62; P[ry$^+$, gsb$^+$]/+* embryos was assessed. In *Df(2R)IIX62/Df(2R)Kr^{SB1}; P[ry$^+$, gsb$^+$]/+*, RP2 duplication is rescued (Fig. 2E, *n*=36 hemisegments). Many, but not all, U and CQ neurons are restored (Fig. 2J); restoration of U and CQ neurons could not be quantified due to the disorganization of the nervous system which results from loss of other neural transcripts in this deficiency combination. *Df(2R)IIX62/Df(2R)IIX62; P[ry$^+$, gsb$^+$]/+* embryos are qualitatively similar to *Df(2R)IIX62/Df(2R)Kr^{SB1}; P[ry$^+$, gsb$^+$]/+* embryos. Since these rescued embryos have the *zipper* phenotype, the results could not be quantified. The implications of the incomplete rescue of U and CQ neurons are discussed in more detail below.

An allelic series of *gsb* mutants

Our results indicate that various *gsb* phenotypes are differentially sensitive to the dosage of functional Gsb protein. We have a series of *gsb* alleles with various amounts of Gsb activity, ranging from *gsb^{P1155}* homozygotes with the most Gsb activity to total loss of *gsb* in the heterozygous deficiency combination. The loss of U and CQ neurons as well as the posterior commissure defects, abundant in *gsb^{P1155}* homozygotes, are most sensitive to a decrease in functional Gsb. The RP2 defect is slightly less sensitive. The cuticle defect appears to be the least sensitive to the dosage of Gsb, as only the deficiency combinations have completely penetrant cuticle defects (Table 1).

To illustrate the effect of Gsb dosage on the CNS phenotype, we have carefully analyzed the percentage of RP2 duplication for a number of combinations of *gsb* mutants (Table 1). Our data indicate that RP2 duplication increases dramatically as more functional Gsb protein is removed. Our results differ somewhat from those of Bhat (1996), who reported 50% RP2 duplication in *Df(2R)IIX62* homozygotes. Although we did not feel that we could accurately assess the *Df(2R)IIX62* homozygotes due to their *zipper* and *gsb* cuticle defects, we find 82±5.0% RP2 duplication in *Df(2R)IIX62/Df(2R)Kr^{SB1}*, 76±4.6% duplication in *gsb^{525}/Df(2R)IIX62* mutants, and 81±4.7% duplication in *gsb^{525}* homozygotes (all three mutant combinations appear to have comparable RP2 phenotypes). Since it was possible to separate the epidermal and neural defects in our analysis, the results

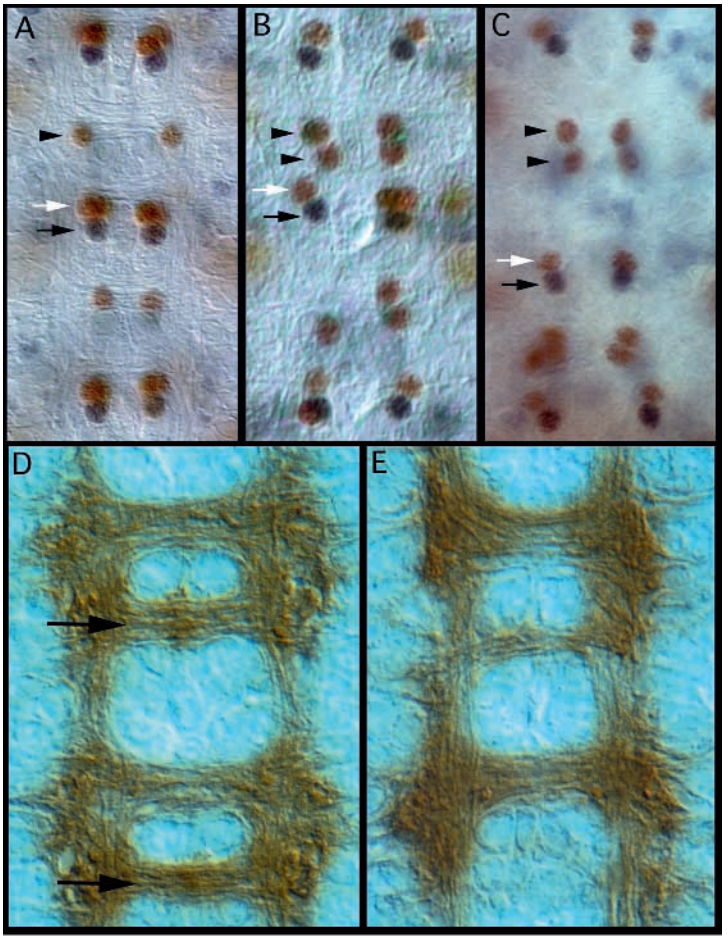

Fig. 3. Neural defects in *gsb^{525}* mutants. The *Y72* enhancer trap line labels pCC neurons (black arrows) and RP3 neurons (out of focus). β-gal (purple) and Eve (brown) expression are shown in *Y72* (A), *gsb^{525} Y72* (B), and *Df(2R)IIX62 Y72* (C) embryos. In A-C, all aCC, pCC and RP2 neurons express Eve (brown), but the pCC neurons also express β-gal, resulting in their purple color. Additional brown cells (out of focus) in the region of aCC and pCC are underlying U neurons. Use of this enhancer trap line confirms that in *gsb^{525}* (B) and *Df(2R)IIX62* (C) homozygotes, RP2 neurons are duplicated (arrowheads), but the aCC (white arrow) and pCC (small black arrow) neurons are normal. Stage 15 wild-type (D) and *gsb^{525}* (E) embryos are labeled with BP102 antibody. In the wild-type embryo (D), posterior commissures (large black arrow) are visible. In *gsb^{525}* mutants (E), the posterior commissure is missing (lower segment) or reduced (upper segment).

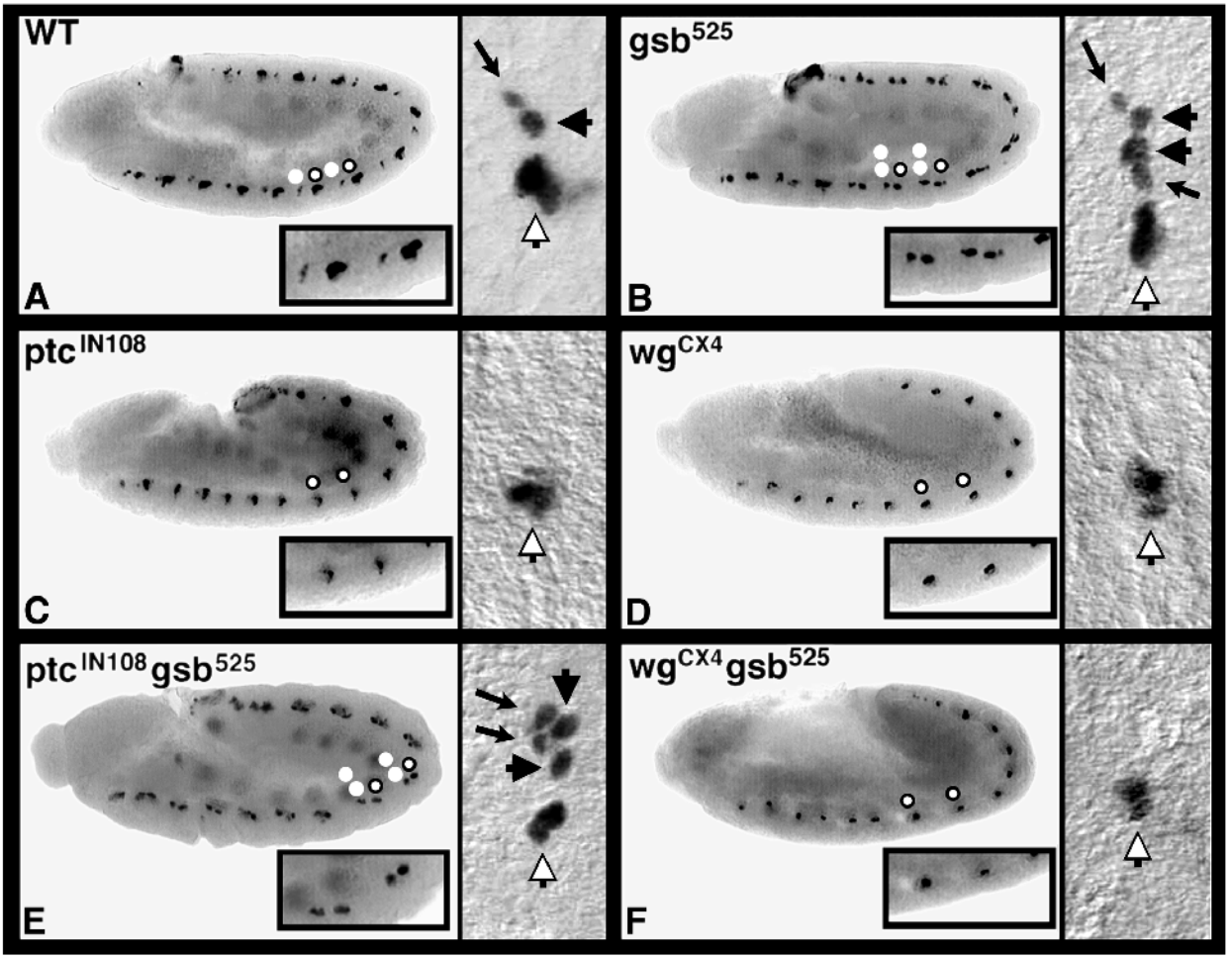

Fig. 4. Analysis of segment polarity gene interactions during CNS patterning. Germ band-extended embryos expressing Eve are shown and oriented with anterior to the left. In each whole-mount embryo, RP2/RP2 sibling neurons are marked by the white dots above them, and the position of the aCC/pCC neuron pairs are marked by the single black-outlined dots above them. Marked segments are magnified in the insets below the embryos. To the right of each embryo, high magnification pictures of a typical hemisegment from germ band-extended embryos of each genotype are shown; in these pictures, black arrowheads mark the RP2 neuron, black arrows mark the RP2 sibling neurons, and white arrowheads point to the aCC/pCC neuron cluster. Wild-type embryos (A) have single RP2 and RP2 sibling neurons in each hemisegment, while *gsb*^{525} (B) and *ptc*^{IN108} *gsb*^{525} double mutant embryos (E) have duplicated RP2 and RP2 sibling neurons. *ptc*^{IN108} (C), *wg*^{CX4} (D), and *wg*^{CX4} *gsb*^{525} mutants (F) lack RP2 and RP2 sibling neurons.

reported here are likely to be more accurate. Also, Bhat (1996) had concluded that the aCC and pCC neurons are duplicated, which could have altered his analysis.

The effect of the dosage of Gsb on the cuticle was also analyzed for various combinations of *gsb* mutants (Table 1). Although *gsb*^{P1155} homozygotes have no cuticle phenotype, *gsb*^{P1155}/*Df(2R)IIX62* mutants and *gsb*^{525} homozygotes have a small percentage of segments with *gsb* cuticle defects (approximately 3%; Table 1). *gsb*^{525}/*Df(2R)IIX62* mutants have a more penetrant cuticle defect (31±2.3% mutant segments, Table 1), and *Df(2R)IIX62/Df(2R)Kr*^{SB1} mutants have a fully penetrant cuticle defect (97±1.1% mutant segments; Table 1). These results indicate that decreasing the level of functional Gsb also results in increased penetrance of the cuticle defect. Although our results illustrate a dosage requirement for Gsb, we did not find cuticle or CNS defects in *Df(2R)IIX62*/+ or *gsb*^{525}/+ heterozygotes. These *gsb* alleles do not show haploinsufficiency.

The rescue results described above may also illustrate the importance of the dosage of Gsb. In *Df(2R)IIX62/Df(2R)Kr*^{SB1}; *P[ry*$^{+}$*, gsb*$^{+}$*]*/+ and *Df(2R)IIX62/Df(2R)IIX62*; *P[ry*$^{+}$*, gsb*$^{+}$*]*/+ flies, the cuticle and RP2 defects, which are least sensitive to the dosage of Gsb, are fully rescued. The U and CQ defects, which are more sensitive to the dosage of Gsb, are not fully rescued. One copy each of the *gsb* transgene and of the endogenous *gsbn* gene are unable to completely rescue the CNS phenotype in *Df(2R)IIX62/Df(2R)Kr*^{SB1}; *P[ry*$^{+}$*, gsb*$^{+}$*]*/+ embryos whereas no CNS phenotype is observed in the presence of one endogenous *gsb* and *gsbn* gene in *Df(2R)IIX62*/+ embryos. The explanation for this apparent discrepancy is again a dosage effect of the Gsb protein since the *gsb* transgene expresses Gsb at a considerably reduced level (<50%) compared to the endogenous *gsb* gene (cf. Fig. 7B,C in Gutjahr et al., 1993).

Interactions among segment polarity genes to specify NB 4-2 fate

The *gsb*^{525} mutant allowed us to specifically define the *gsb* neural phenotype without the complication of epidermal

defects. This mutant has also allowed us to look at the interactions among segment polarity genes during CNS patterning. We have focused on the patterning of NB 4-2. The first ganglion mother cell (GMC) produced by NB 4-2 divides to produce the RP2 neuron and the RP2 sibling (reviewed by Doe, 1992). Although *gsb* mutants have duplicated RP2 neurons (Patel et al., 1989; Figs. 2B-D and 4B), *wg* mutants lack RP2 neurons (Patel et al., 1989; Fig. 4D). Previous studies have indicated that the RP2 defects seen in *wg* and *gsb* mutants can be attributed to cell fate mis-specification at the NB level (Chu-LaGraff and Doe, 1993; Skeath et al., 1995). NB fate is specified prior to NB delamination, during the patterning of the ventral neuroectoderm. Wg protein, which is expressed in row 5 neuroectodermal cells, acts nonautonomously to control the fate of adjacent row 4 neuroectodermal cells. The lack of RP2 neurons in *wg* mutants is due to a transformation of NB 4-2 to the NB 3-2 fate (Chu-LaGraff and Doe, 1993). *gsb,* which is expressed in row 5 cells, specifies row 5 NB identity (Gutjahr et al., 1993; Skeath et al., 1995). Row 5 NBs in *gsb* mutants lose expression of some, but not all, row 5 cell markers (Skeath et al., 1995; our data, not shown), indicating that changes occur at the NB level. Furthermore, the fact that the sibling of RP2 is duplicated in *gsb* mutants supports a role for Gsb in patterning at the NB level. When GMC 4-2a divides to produce RP2 and RP2 sibling, both of these neurons initially express Eve (the expression of Eve in the RP2 sibling is turned off quickly). Our analysis, as well as the Bhat (1996) analysis, has shown that in *gsb* mutants, both the RP2 and RP2 sibling neurons are duplicated (Fig. 4B). These data indicate that the entire NB 4-2 lineage is duplicated.

In order to understand the functions of Wg and Gsb in NB 4-2 specification, we constructed wg^{CX4} gsb^{525} and wg^{IL114} gsb^{525} recombinants. *wg gsb* double mutants lack RP2 neurons, displaying the *wg* phenotype (Fig. 4F). Based on these results, we propose a model for the specification of NB 4-2 cell fate (Fig. 6A,B). Secreted Wg protein confers the NB 4-2 fate while Gsb acts to prevent the row 5 cells, which secrete Wg, from responding to the Wg signal. In a *wg gsb* mutant, the *wg* mutation is epistatic to the *gsb* mutation, as the NB 4-2 cell fate cannot be specified without the Wg signal (Fig. 6A). We emphasize that during the time of NB specification, *wg* expression is not altered in *gsb* mutants (Fig. 5B,C), and Gsb

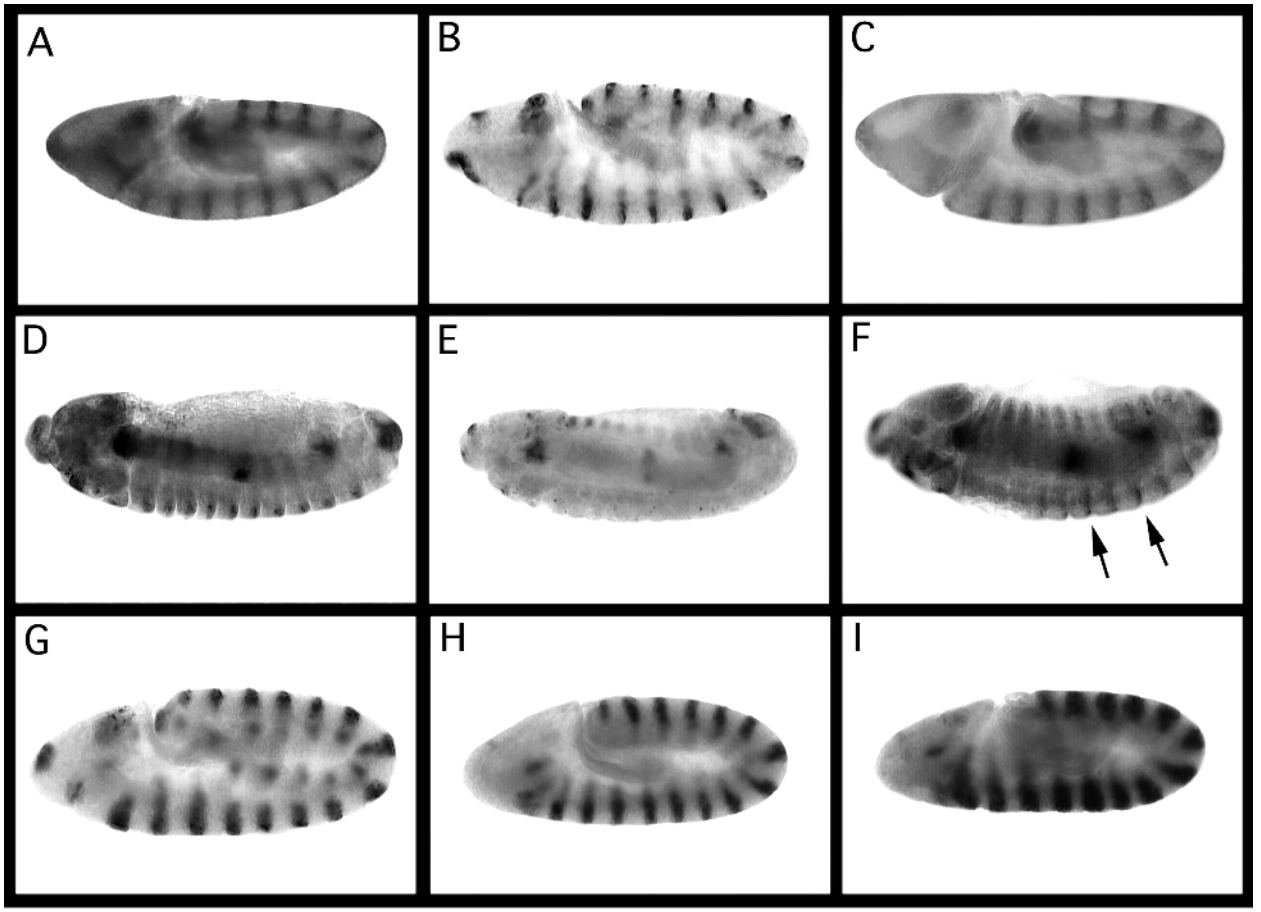

Fig. 5. *wg* and *gsb* expression in segment polarity mutants. Wg expression (protein in A and C, transcript in B) in *Df(2R)IIX62* homozygotes (B) and gsb^{525} mutants (C) in the ventral neuroectoderm is comparable to that found in wild-type embryos (A) through the period when NB 4-2 is determined (stage 9 depicted in A-C). Following the period of NB 4-2 determination, during the period of *wg-gsb* autoregulation (stages 11 through 13), *wg* transcripts are not detected in the epidermis of *Df(2R)IIX62* homozygote mutants (stage 13 depicted in E; compare to wild-type Wg protein expression in D). At an equivalent time point, Wg protein expression can be detected at reduced levels in the epidermis of gsb^{525} mutants (F, arrows). During the time of NB 4-2 determination, *wg* RNA expression expands anteriorly in ptc^{IN108} mutants (G; compare to wild-type Wg expression in A). At the same stage, *gsb* RNA expression expands anteriorly in ptc^{IN108} mutants (I; compare to wild-type *gsb* expression in H).

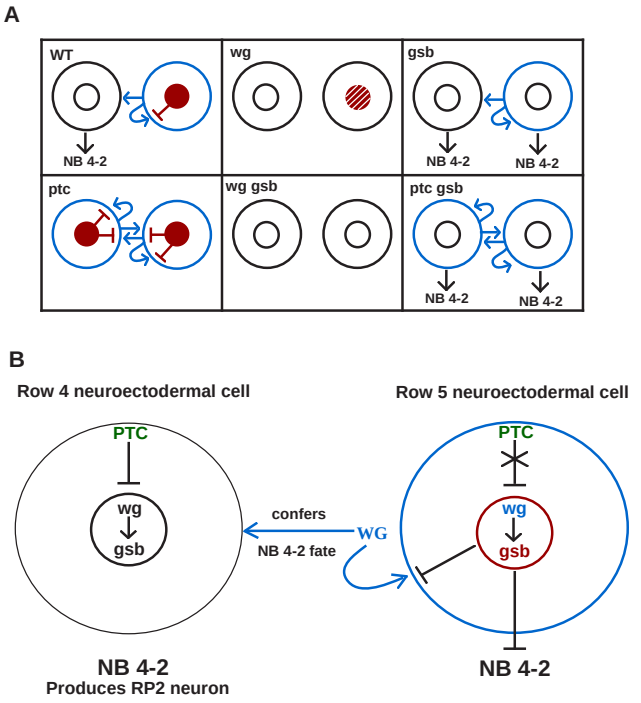

Fig. 6. Segment polarity gene interactions during patterning of the ventral neuroectoderm. For the sake of simplicity, the genetic interactions between segment polarity genes for a single pair of cells are shown, but we imagine that the interactions actually occur between small patches of cells within the neuroectoderm. The action of neurogenic loci within each group of cells results in the production of only a single NB from each group. (A) *gsb* (brown) and *wg* (blue) interactions for the patterning of NB 4-2 are shown in different mutant backgrounds. In each panel, the left cell represents a row 4 neuroectodermal cell, and the right cell represents a row 5 neuroectodermal cell. In wild-type embryos, Wg is secreted from the row 5 cell, conferring the NB 4-2 fate on the adjacent cell. Gsb is expressed in the row 5 cell and acts to antagonize the NB 4-2 specifying function of Wg, preventing the row 5 cell from taking on the NB 4-2 fate. In *wg* or *wg gsb* mutants, the Wg signal is absent, and NB 4-2 fate is not specified. In the *wg* mutant panel, *gsb* expression is drawn with hatchmarks, representing the gradual decline and eventual loss of *gsb* transcript during the period of NB 4-2 specification (the important point still being that the Wg signal is absent, and no NB 4-2 is specified). In *gsb* and *ptc gsb* mutants, Gsb does not antagonize the Wg signal in row 5, so both the row 4 and row 5 cells take on the NB 4-2 fate. In *ptc* mutants, *wg* and *gsb* are expressed ectopically in the row 4 cell. Gsb expression in both the row 4 and row 5 cells antagonizes the NB 4-2 specifying Wg signal, and neither cell becomes NB 4-2. (B) The model for NB 4-2 specification is drawn in more detail. During the patterning of the ventral neuroectoderm, Wg, expressed in row 5 cells, specifies NB 4-2 fate in row 4 cells and, at the same time, maintains *gsb* expression in row 5. In row 5, Gsb antagonizes the NB 4-2 specification function of secreted Wg, preventing row 5 cells from taking on a NB 4-2 fate. In row 4, Ptc represses the expression of *wg*, and consequently *gsb*. Since row 4 cells do not express *gsb*, they can receive the Wg signal and take on the NB 4-2 fate. In row 5 cells, Ptc repression of *wg* is prevented, presumably by the reception of the Hedgehog signal (reviewed by Perrimon, 1994). Although Gsb antagonizes Wg signaling during the patterning of NB 4-2, at a later stage, Gsb maintains *wg* expression, which is necessary for a wild-type cuticle.

is not required to maintain the expression of the Wg signal which specifies NB 4-2 fate. The phenotype of *Hs-gsb* embryos supports our model for the patterning of NB 4-2. When *gsb* is expressed everywhere under control of the heat shock promoter, row 4 cells express row 5 markers (Skeath et al., 1995), and RP2 neurons do not form (Li and Noll, 1994; Zhang et al., 1994).

ptc mutants lack RP2 neurons (Patel et al., 1989; Fig. 4C), indicating that Ptc could also play a role in the specification of NB 4-2. Two explanations could explain the loss of RP2 neurons in *ptc* mutants: (1) Ptc could be required in row 4 for proper reception or interpretation of the Wg signal which confers NB 4-2 fate, or (2) the ectopic expression of *wg* and *gsb* in *ptc* mutants in the ventral neuroectoderm (see below) could disrupt NB patterning and result in loss of RP2 neurons.

Beginning during stage 8, Wg controls *gsb* expression (Li and Noll, 1993; Li et al., 1993). The Wg domain expands anteriorly in *ptc* mutants (Martinez Arias et al., 1988; Bejsovec and Wieschaus, 1993; Fig. 5G), and since *gsb* expression is under the control of Wg at this time, the Gsb domain also expands in *ptc* mutants (Hidalgo, 1991; Fig. 5I). In a wild-type embryo, the secretion of Wg from row 5 cells is not sufficient to generate expression of *gsb* within row 4 cells, indicating that the ectopic expression of *gsb* in a *ptc* mutant depends on the actual expression of *wg* within the cells ectopically expressing *gsb*.

We carefully reexamined the time point at which *gsb* expression expands in *ptc* mutants and found that it begins during stage 8, while the ventral neuroectoderm is being patterned (Fig. 5I), at the time when Wg acts to determine NB 4-2 fate (Chu-LaGraff and Doe, 1993), and prior to the delamination of NB 4-2 (reviewed by Doe, 1992). Since ectopic *gsb* expression in *ptc* mutants occurs at a stage when it could be relevant to NB 4-2 patterning, the lack of RP2 neurons in *ptc* mutants could be due to the expansion of the *gsb* domain (Fig. 6A). *gsb* expression data therefore favor the second explanation for the *ptc* RP2 defect.

Analysis of the ptc^{IN108} gsb^{525} double mutants provides additional support for this second explanation; *ptc gsb* mutants have duplicated RP2 neurons (Fig. 4E). This phenotype was analyzed at stage 11, when the overall disorganization of the embryo which eventually results from loss of *ptc* is minimal. The *gsb* phenotype of the *ptc gsb* mutant indicates that RP2 neurons can be formed in the absence of Ptc. Ptc is not necessary for the proper reception or interpretation of the NB 4-2 specifying function of Wg, thus ruling out the first explanation of the *ptc* mutant phenotype. Our results indicate that Ptc normally functions to limit *wg*, and consequently *gsb* expression, allowing proper NB 4-2 specification (Fig. 6A,B). The *ptc* phenotype is therefore somewhat similar to the loss of RP2 neurons in *Hs-gsb* embryos (see above).

DISCUSSION

Two *gsb* alleles separate the function of *gsb* in cuticle and CNS patterning

For a number of years, researchers have attempted to obtain separate *gsb* and *gsbn* alleles. Here, we report the characterization of the first two *gsb* alleles. These alleles, as well as *gsb* rescue experiments, have allowed us to determine if the previ-

ously reported defects associated with the *gsb* deficiency alleles (Patel et al., 1989) can be attributed to a loss of *gsb*. These new *gsb* mutants have neural defects but lack epidermal defects. Genetic separation of the epidermal and CNS patterning functions of *gsb* makes analysis of the *gsb* CNS defects more straightforward.

The gsb^{525} and gsb^{P1155} defects include: duplication of RP2 neurons (Figs 2C,D, 3B, 4B), loss of U and CQ neurons (Fig. 2H,I), and loss or reduction of the posterior commissure (Fig. 3E). These defects can be rescued in the deficiency mutants with the *gsb* rescue construct (Fig. 2E,J). Our increased ability to positively identify cells in the two new *gsb* mutants and the use of the Y72 enhancer trap (Fig. 3A-C) has allowed us to make a correction of the previously reported *gsb* phenotype (Patel et al., 1989): the aCC and pCC neurons are not duplicated.

We have focused on the genetic characterization of the two new *gsb* alleles. Future studies will more precisely address the molecular nature of these alleles. The possibility that a truncated Gsb^{525} protein containing a paired-domain but no homeodomain (Fig. 1C) could have partial function is particularly interesting in light of recent studies which have shown that both the paired- and homeodomains are necessary for *prd* function in epidermal patterning (Bertuccioli et al., 1996; Fujioka et al., 1996; Miskiewicz et al., 1996; L. Xue and M. Noll, unpublished). Since a number of *Pax* genes have a paired-domain, but no homeodomain (reviewed by Noll, 1993), it seems plausible that such a truncated protein might have partial function.

An invertebrate model for the study of *Pax* gene dosage effects

The vertebrate *Pax* genes are transcriptional regulators which were isolated through sequence homology to the paired-domain of the *Drosophila prd*, *gsb* and *gsbn* genes (reviewed by Noll, 1993). Interestingly, many *Pax* mutations, including *Pax 6* alleles, are haploinsufficient (reviewed by Gruss and Walther, 1992; Chalepakis et al., 1993). Recently, Schedl et al. (1996) showed that mice with extra copies of *Pax 6* have severe eye abnormalities, demonstrating once again how critical the dosage of *Pax* genes can be.

Although we did not find evidence for haploinsufficiency of *gsb*, our data illustrate that the level of functional Gsb protein is of extreme importance. The U, CQ and posterior commissure defects, abundant in our weakest *gsb* mutant, gsb^{P1155} (Fig. 2I), are most sensitive to loss of Gsb. The RP2 neural defect is slightly less sensitive, but decreasing the amount of functional Gsb results in an increase in RP2 neuron duplication (Table 1). The cuticle defect, undetectable in gsb^{P1155} mutants, is the least sensitive to decreases in the amount of functional Gsb, but as more Gsb activity is removed, the cuticle defect becomes more penetrant (Table 1). Interestingly, Gsb also exhibits strong dosage effects when substituting for Prd functions in *prd*-Gsb evolutionary alleles (Xue and Noll, 1996). Our results indicate that we have identified an invertebrate model for the study of paired-box gene dosage effects.

Several explanations for *Pax* gene haploinsufficiency have been suggested (Read, 1995). Schedl et al. (1996) discuss how these explanations might be applicable to *Pax 6* haploinsufficiency. They argue that the most likely model to account for *Pax 6* gene haploinsufficiency is the existence of many target gene binding sites with varying affinities for Pax6 protein. A different spectrum of target genes might be expressed depending on the amount of Pax6 that can be found within a given cell. If the level of Pax6 is lowered, the spectrum of target genes that are able to respond is altered. This model could apply to our results. Perhaps the neural target genes of Gsb have the lowest affinity binding sites, such that a slight drop below one dose of wild-type Gsb activity results in a neural phenotype. The target genes responsible for epidermal patterning may have higher affinity binding sites which could bind Gsb protein even when the Gsb levels have dropped to a point where neural phenotypes are detectable.

In the hypothesis described above, a truncated Gsb^{525} protein (Fig. 1C) could be considered to have decreased function. An alternative explanation of our results would be that the homeodomain, which would be missing in the truncated Gsb^{525} protein, is necessary for the neural patterning functions of Gsb, but is not needed for proper patterning of the epidermis. We do not favor this explanation for several reasons. Cuticle phenotypes can be detected in $gsb^{525}/Df(2R)IIX62$ mutants, indicating that a truncated protein without a homeodomain is not sufficient for proper epidermal patterning. Also, detection of neural, but not epidermal defects in gsb^{P1155} mutants, which likely have lower levels of functional Gsb protein, favors the explanation that Gsb^{525} protein has an overall reduction in function. Although we assume that gsb^{525} mutants synthesize largely truncated Gsb^{525} protein, their hypomorphic nature may very well result from a small fraction of readthrough at the stop codon (Fig. 1C), generating low levels of full-length Gsb protein that we cannot detect.

The role of *gsbn*

Some researchers had initially wondered whether *gsb* and *gsbn* would have redundant or overlapping functions. However, Li and Noll (1994) determined that the functions of these genes, which appear to have homologous protein functions, have evolved through changes in *cis*-regulatory elements. For example, although we demonstrate that Gsb, which is expressed in the ventral neuroectoderm, plays a role in NB patterning and RP2 specification, *gsbn* is not expressed in the ventral neuroectoderm during the time of NB specification and hence cannot play a role in neural specification at this level (Gutjahr et al., 1993).

Although the function of Gsb in RP2 specification is completed at the level of NB patterning, in other cases, Gsb may only act to initiate a neural specification program which would be upheld by target genes that are expressed after the NB has divided. Since Gsb is thought to activate Gsbn expression in Gsb-expressing NB lineages (Gutjahr et al., 1993; Zhang et al., 1994), Gsbn may function as one of these target genes. Our results uphold this hypothesis in a number of ways. First, Gsbn expression is drastically reduced in the hypomorphic allele gsb^{525}, suggesting that Gsb is responsible for regulating *gsbn* expression (not shown). Furthermore, we find incomplete rescue of U and CQ neurons in *Df(2R)IIX62* homozygotes and $Df(2R)IIX62/Df(2R)Kr^{SB1}$ heterozygotes (Fig. 2J) possessing one copy of the *gsb* transgene. As discussed earlier, this incomplete rescue may simply result from subnormal expression levels of *gsb* from the *gsb* transgene. Alternatively, it may indicate that *gsbn* is required for a complete set of U and CQ neurons, as *gsbn* is deleted in

Df(2R)IIX62 homozygotes (Fig. 1A) and Gsbn expression is strongly reduced in *Df(2R)IIX62/Df(2R)Kr^{SB1}* embryos carrying even two copies of the *gsb* transgene (Gutjahr et al., 1993). Therefore, loss of *gsbn* expression may also contribute to the incomplete rescue of U and CQ neurons in these embryos. Consistent with this, we find expression of Gsbn in the U and CQ neurons (data not shown). The discovery of *gsbn* mutants would clarify this issue.

Novel genetic interactions between *gsb* and *wg*

Our results have allowed us to gain an understanding of the interactions between *gsb*, *ptc*, and *wg* for the patterning of NB 4-2. We independently came to conclusions similar to those reported by Bhat (1996), but our results extend this analysis. A number of previous studies, including the Bhat (1996) analysis, used *Df(2R)IIX62* homozygote embryos. In our study, we use the *gsb^{525}* allele; we can therefore be certain that the genetic interactions for patterning NB 4-2 are truly occurring between *gsb*, *wg*, and *ptc*, and have nothing to do with any other genes removed by the *gsb* deficiency alleles. More significantly, our interpretations of neural phenotypes avoid possible complications due to *gsb* cuticle defects, and we can analyze interactions for NB patterning somewhat separately from those for epidermal patterning.

The genetic interactions among segment polarity genes for patterning the ventral neuroectoderm are illustrated in Fig. 6B. In summary, Wg specifies NB 4-2 fate and, at the same time, maintains *gsb* expression. In row 5, Gsb antagonizes the function of secreted Wg, preventing the row 5 cells, which secrete Wg, from taking on the NB 4-2 fate. Ptc represses the expression of *wg*, and consequently that of *gsb*, in row 4 cells. Since row 4 cells do not express *gsb*, they can receive the Wg signal and take on the NB 4-2 fate. Therefore, during NB 4-2 patterning, Gsb antagonizes Wg signaling. In contrast, at a later point in development, during the phase of *wg-gsb* autoregulation, Gsb acts to maintain *wg* expression, which is responsible for the specification of naked cuticle (Li and Noll, 1993). Thus, the early genetic interactions demonstrated to occur between *gsb* and *wg* for specifying NB 4-2 (this analysis; Bhat, 1996) are different from the previously reported epidermal patterning interactions between *gsb* and *wg* during stages 11 through 13. The mechanism by which Gsb antagonizes Wg signaling in CNS development is unknown. Perhaps Gsb also antagonizes Wg signaling during epidermal patterning in a manner which has yet to be uncovered.

The temporal aspects of the different *gsb/wg* interactions described above are very important. Previous models (Skeath et al., 1995) had proposed that during NB patterning, *gsb* positively regulates *wg*. However, such a positive interaction has not yet been demonstrated to occur before stage 11, when *wg* expression fades in *gsb* mutants (Li and Noll, 1993; Fig. 5E,F). The timing of this positive *wg-gsb* autoregulation also relates to another problem with the Skeath et al. (1995) model. This model, as well as a model proposed by Zhang et al. (1994), had indicated that in *gsb* mutants, row 5 NBs are transformed to row 3 NBs. However, *gsb* mutants still express Wg (Fig. 5B,C), a row 5 marker which is not normally expressed in row 3 cells. Therefore, analysis with NB markers can sometimes lead to inconsistent interpretations.

The discovery that Gsb can function to repress Wg signaling could have important implications for understanding the role of *Pax* and *Wnt* genes in the patterning of the vertebrate hindbrain. Previously, researchers had proposed that *Pax-2* is necessary for *Wnt-1* transcription (Krauss et al., 1992; Rowitch and McMahon, 1995). Our results indicate, however, that *gsb/Pax* gene products may also antagonize the Wg/Wnt signaling functions, preventing cells which secrete the Wg/Wnt signal from taking on the fate conferred by the signal.

An interesting parallel to the *gsb/wg* interactions described here can be found in the patterning of the wing margin (Couso et al., 1994). During patterning of the wing margin, Wg, secreted from the edge cells, signals adjacent marginal cells to express *achaete* (*ac*). However, the edge cells which secrete Wg do not express *ac*. In these edge cells, Wg regulates *cut (ct)* expression, and Ct blocks the Wg signal which would turn on *ac* expression. Thus, in both the patterning of the wing margin, as well as in the patterning of NB 4-2, a secreted signaling molecule confers a particular cell fate; at the same time, the signaling molecule regulates expression of a transcription factor within the cells which secrete the signal, effectively preventing these cells from taking on the fate conferred by the signal. Such a theme may be common to many signal transduction and cell patterning systems.

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