The patched signaling pathway mediates repression of gooseberry allowing neuroblast specification by wingless during Drosophila neurogenesis

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SUMMARY

The Drosophila signaling molecule Wingless (Wg) plays crucial roles in cell-cell communications during development. In the developing nervous system, a previous study has shown that Wg acts non-autonomously to specify the fate of a specific neuronal precursor, NB4-2 (Q. Chu-LaGraff and C. Q. Doe (1993) Science 261, 1594-1597). The lack of autocrine specification of NB4-2 in Wg-expressing cells suggests that the response to Wg is spatially restricted, presumably through the activity of the Wg-receptor. I show that two other proteins, a transcription factor Gooseberry (Gsb) and a transmembrane protein Patched (Ptc), participate in the Wg-mediated specification of NB4-2 by controlling the response to the Wg signal. In gsb mutants, Wg-positive NB5-3 is transformed to NB4-2 in a Wg-dependent manner, suggesting that Gsb normally represses the capacity to respond to the Wg signal. In ptc mutants, Gsb is ectopically expressed in normally Wg-responsive cells, thus preventing the Wg response and consequently the correct specification of NB4-2 does not take place. This conclusion is supported by the observation that NB4-2 can be specified in gsb;ptc double mutants in a Wg-dependent manner. Moreover, ectopic expression of Gsb from the hsp70-gsb transgene also blocks the response to the Wg signal. I propose that the responsiveness to the Wg signal is controlled by sequential negative regulation, ptc→gsb→Wg receptor. The timing of the response to Gsb suggests that the specification of neuroblast identities takes place within the neuroectoderm, prior to neuroblast delamination.

Key words: patched, gooseberry, wingless, neuroblast, signaling, neurogenesis

INTRODUCTION

The question of how the central nervous system (CNS) is generated in eukaryotes is of great significance. In the fruit fly Drosophila, during neurogenesis about 30 heterogeneous Neuroblast (NB) stem cells delamate from the bipotential neuroectoderm in each hemisegment (Bate, 1976; Hartenstein and Campos-Ortega, 1984; Cabrera, 1992). Once the identities of these neuroblasts are established, with a few exceptions, these neuroblasts function much like stem cells, undergoing a series of asymmetric cell divisions to self-renew and to produce a chain of ganglion mother cells (GMCs). The GMCs divide only once to give rise to two cells, which differentiate into specific neurons. When the differentiation of the embryonic CNS is complete, each hemisegment is believed to consist of about 250 distinct and highly specialized neurons.

While much is known about the formation of neuroblasts (see Campos-Ortega, 1993; Cabrera, 1992), the genetic network that specifies individual neuroblast identities is not well understood. To address this question, I have selected one of the well-studied neuronal lineages, the RP2 lineage (c.f., Thomas et al., 1984; Chu-LaGraff and Doe, 1993; Bhat and Schedl, 1994; Bhat et al., 1995; Chu-LaGraff et al., 1995). The RP2 neuron is a motoneuron and innervates muscle number 2 of the dorsal musculature. This neuron originates along with its sibling cell from the first GMC of NB4-2 and occupies the anterior commissure along with several other RP neurons (Thomas et al., 1984; see Bhat and Schedl., 1994; Bhat et al., 1995; Chu-LaGraff et al., 1995). NB4-2 itself is formed during the second wave of neuroblast delamination (therefore an S2 neuroblast) in stage 9 (~4.5 hours old) of embryogenesis (see Doe, 1992). One class of genes likely to play a crucial role in the specification of NB4-2 and other neuroblasts are those involved in the segmentation process. For example, previous results show that mutations in three such genes, patched (ptc), gooseberry (gsb) and wingless (wg), affect the specification of the RP2 lineage (Patel et al., 1989). The ptc gene encodes a transmembrane protein (Hooper and Scott, 1989; Nakano et al., 1989), wg encodes a signaling molecule (reviewed in Klingensmith and Nusse, 1994) and gsb encodes a transcription factor (Bopp et al., 1986; Baumgartner et al., 1987).

While a loss of Wg or Gsb activity during segmentation results in a loss of naked cuticle, a similar loss of Ptc activity results in a mirror-image duplication of segment boundaries and adjacent cuticle replacing the remainder of the segment (see Lindsey and Zimm, 1982). More recent studies of Bejovec and Wieschaus (1993) and Schuske et al. (1994) indicate that, during segmentation, Wg is repressed by Ptc in the ectodermal cells anterior to row 9 (corresponds to row 5 neuroblasts). Thus in ptc mutants, Wg expression is expanded into cells anterior to row 9. In row 9 ectodermal cells, however, Wg is uncoupled from Ptc regulation...
and both are co-expressed. The genetic evidence suggest that, in these row 9 cells, the Hedgehog (Hh) signaling molecule interacts with Ptc to relieve the repression of Wg by Ptc (see review by Klingensmith and Nusse, 1994). The requirement of these segment polarity genes during neurogenesis is less thoroughly understood. For instance, in the developing nervous system, a loss of function for ptc leads to missing Even-skipped (Eve)-positive RP2 motoneurons (Patel et al., 1989). However, the reason for this loss of RP2 neurons in ptc mutants, the requirement of Ptc during neuroblast delamination and/or specification, or the interaction of ptc with other segmentation genes such as wg and gsb, is not known. Similarly, while it is known that the loss of RP2 neurons in wg mutants is due to mis-specification of NB4-2 identity (Chu-LaGraff and Doe, 1993), how the Wg signaling pathway relates to the Ptc signaling pathway or Gsb during neurogenesis is not known. In addition to this, since two of these genes, ptc and wg, are expected to function in the signal transduction pathways (c.f., Klingensmith and Nusse, 1994; Martinez Arias, 1994), analysis of these genes and their interactions would also help to understand how cell-cell signaling specifies different neuroblast identities.

By contrast to a loss of function for ptc or wg, a loss of function for gsb (Bopp et al., 1987; Baumgartner et al., 1987) results in a duplication of the RP2 neurons (Patel et al., 1989). While duplication of the RP2 neurons in gsb mutants is due to NB5-3 changing to NB4-2 (Skeath et al., 1995; this report), the reason for this fate transformation in gsb mutants is not known. Furthermore, while Gsb is required for maintaining Wg expression during segmentation (Li and Noll, 1993; see review by Klingensmith and Nusse, 1994), the relationship of Gsb to Wg and Ptc signaling pathways during neurogenesis is less clear.

To address these questions, I have analyzed ptc and gsb mutations and interactions of these two with wg during the specification of NB4-2, the precursor neuroblast of the RP2 neuron. My results show that Ptc is required for the formation, as well as for the specification of NB4-2 identity. The mis-specification of NB4-2 in ptc mutants is due to the de-repression of Gsb within the neuroectoderm from which this neuroblast delaminates. The de-repressed Gsb restricts the capacity of these neuroectodermal cells to respond to the Wg signal, resulting in the mis-specification of NB4-2. However, the elimination of Gsb from ptc embryos allows reception of the Wg signal and the correct specification of the NB4-2 identity. In gsb mutants, the Wg-positive NB5-3 is able to receive the autocrine Wg signal. This reception of the Wg signal transforms NB5-3 to a NB4-2 cell resulting in the duplication of the RP2 lineage. In addition to these results, I find that ectopic expression of Wg in the neuroectoderm using an inducible promoter causes duplication of the RP2 lineage in wild type and triplication in gsb mutants. However, the duplication of the RP2 lineage by ectopic expression of Wg can be suppressed by a simultaneous ectopic expression of Gsb. These results indicate that the Wg signal can specify NB4-2 identity only in the absence of Gsb and thus uncover a novel function for Gsb in the Wg signaling pathway—repressing the competence of cells to respond to the Wg signal.

MATERIALS AND METHODS

Fly strains and genetics

ptc^{IN108} is a null allele and ptc^{H84} is a lacZ enhancer-trap null allele of ptc. The gsb mutation is a deficiency that removes both gsb-distal and gsb-proximal genes. The wg alleles used were wg^{G22}, wg^{C34} and wg^{l144}. wg^{G22} is a strong allele, wg^{C34} is a null allele and wg^{l144} is the temperature-sensitive allele of wg. All the fly strains, except ptc^{H84} allele, were balanced using lacZ-marked second or third chromosome ‘blue’ balancers. This allows unambiguous identification of homozygous mutant embryos. The hh^{H84} is an enhancer-trap viable allele of huckebein and is a NB4-2-specific marker (see Chu-LaGraff et al., 1995). For ubiquitous expression of wg, a transgenic line carrying a full-length wg cDNA under the hs70 promoter (Hs-wg) was used (see Noordermeer et al., 1992). For ubiquitous expression of gsb, a transgenic line carrying a full-length gsb-d cDNA under the hs70 promoter (Hs-gsb) was used (see Li and Noll, 1994). Except for the Hs-wg and hh^{H84}, which are on the third chromosome, all other mutations are on the second chromosome. See Lindsley and Zimm (1982) for more details on these mutations. The double mutants between second and third chromosome mutations were constructed by standard genetics. The double mutants between second chromosome mutations were constructed by genetic recombination. The double mutant ptc^{H84}/gsb has been described previously (Hooper, 1995). All experiments were done either at 22°C or at 25°C except for the heat-shock experiment (see below).

Embryo collection and fixation

Embryos were collected on apple juice agar plates at 22°C. They were dechorionated using 40% bleach and fixed by the N-heptane, formaldehyde method as described previously (Bhat and Schell, 1994; Bhat et al., 1995, 1996). The length of fixation was dependent on the specific antibody used for immunostaining (see below).

Immunostaining

To determine the expression pattern of Ptc during neurogenesis and for examining whether the Ptc-positive neuroblasts are formed in ptc mutants, the lacZ enhancer-trap ptc^{H84} allele was used. Heterozygous (ptc^{H84}/+4) and homozygous (ptc^{H84}/ptc^{H84}) embryos were stained with β-gal antibody (Promega; 1:500 dilution; 25 minutes fixation). Unlike the Ptc protein, which is an integral membrane protein, the lacZ enhancer-trap Ptc protein accumulates in the nucleus and hence more sensitive to detection. To examine whether the NB4-2 identity is specified in ptc mutants, ptc^{IN108} embryos carrying the NB4-2-specific enhancer-trap line hh^{H84} (see Doe, 1992; Chu-LaGraff et al., 1995) were stained with β-gal antibody. Homozygous ptc embryos (ptc^{IN108}/ptc^{IN108}) were identified by their lack of lacZ-CyO-specific β-gal expression. To monitor Hkb expression in gsb mutants, embryos were stained with β-gal antibody to visualize Hkb expression and monoclonals against Gsb-d and Gsb-p to identify gsb embryos (1: 5; 25 minutes fixation). For Runt staining, a mixture of monoclonals against Runt was used (1:10, 15 minutes fixation). For Eve staining, a rabbit polyclonal antibody was used (1:2000; 22 minutes fixation). For examining a mature RP2 neuron, mAb 22C10, a monoclonal raised against a membrane protein, was used (1:25; 40 minutes fixation). The Runt whole-mount in situ were performed as described previously (Tautz and Pfeile, 1989). For examining the expression of Wg, a rabbit polyclonal antibody against Wg (1:200; 35 minutes fixation) was used.

Staging of embryos

Embryos were staged according to Wieschaus and Nusslein-Volhard (1986) at room temperature (~22°C). The delamination of S1 (stage 8; ~4 hours old), S2 (stage 9; ~4.5 hours old) and S3 (stage 10; ~5 hours old) neuroblasts were also used as additional criteria for staging.

Heat-shock experiment

To express Wg ectopically during development, a transformant line carrying a full-length wild-type wg-cDNA fused to a hs70 promoter was used (see Noordermeer et al., 1992). While a 15 minute heat shock induced moderate levels of Wg, 20 minute heat shock induced a high level of Wg in all cells. This Wg protein persists up to ~50 minutes and after that fades away. Additional characteristics of this line have been
described previously (Noordermeer et al., 1992). Embryo collections were done at 22°C for 15 minutes. Early cleavage stage embryos (~15 minutes old) were then hand picked to ensure the accuracy of the developmental stage and aged for different durations. Just prior to heat shock, these embryos were again examined for their developmental stages and appropriately staged embryos (see Fig. 7 and text) were again hand picked for heat shock. The heat shock was performed for 20 minutes at 37°C by immersing the embryos in prewarmed halocarbon oil. These embryos were then washed quickly with heptane, dechorionated and fixed with heptane-formaldehyde mixture and stained with Eve antibody. To examine the expression of NB4-2 marker Hks5953, Hs-wg embryos carrying the hkb5953 chromosome were treated as above and stained with β-gal antibody. They were then washed quickly with heptane, dechorionated and fixed with heptane-formaldehyde mixture and stained with Eve antibody. To examine the expression of NB4-2 marker Hks5953, Hs-wg embryos carrying the hkb5953 chromosome were treated as above and stained with β-gal antibody. Non-heat-shocked Hs-wg embryos or heat-shocked wild-type embryos did not show any particular defect.

RESULTS

Patched is expressed in NB4-2 and its precursor neuroectoderm

The RP2 neuron is a motoneuron and innervates muscle number 2 on the dorsal musculature. It is formed from the first ganglion mother cell (GMC-1) of NB4-2, along with its sibling cell. While it is known that loss of RP2 neurons in wg mutants is due to an altered NB4-2 identity (Chu-LaGraff and Doe, 1993) and duplication of the RP2 neurons in gsb mutants is due to a second neuroblast, NB5-3, changing to NB4-2 (Skeath et al., 1995; also this report), the reason for the loss of RP2 neurons in ptc mutants was not known. The loss of Ptc function could affect the formation and/or the specification of NB4-2, GMC-1 or the RP2 neuron itself. In order to determine this, initially I examined in which of these cells (NB4-2, GMC-1 or RP2/sib) Ptc is expressed in this lineage. For this purpose, a lacZ enhancer-trap ptc mutant allele, ptcH84 (Hooper, 1995), was used. Since ptcH84 is a phenotypic null allele, heterozygous embryos (ptcH84/+) were examined with β-gal antibody for the expression pattern of Ptc. These results are presented in Fig. 1. As shown in this figure, Ptc is expressed in the neuroectoderm from which NB4-2 is delaminated (Fig. 1A), as well as in NB4-2 itself (Fig. 1B). However, Ptc is not expressed in GMC-1, newly formed RP2/sib cells, or mature RP2 neurons, as judged by double staining ptcH84/+ embryos with β-gal and Eve antibodies (Eve is expressed in GMC-1 but not in NB4-2; it continues to be expressed in RP2 but disappears from sib, see Bhat and Schedl., 1994; Bhat et al., 1995; also below). The posterior boundary of the Ptc expression domain was confirmed by double staining ptcH84/+ embryos with antibodies against β-gal (for detecting Ptc) and Wg or β-gal and Engrailed (En). Wg is expressed in row 5 neuroblasts and their precursor neuroectoderm while En is expressed in rows 6 and 7, and NB1-2 of row 1 and their precursor neuroectoderm (see Fig. 1, upper right line drawing; see Doe, 1992). The anterior boundary of the Ptc expression domain was determined by double staining ptcH84/+ embryos with β-gal and Runt (which is expressed in a subset of row 2 neuroblasts) as well as using morphological landmarks such as tracheal pits (which are formed just anterior to row 2 neuroblasts, see Fig. 1, line drawings). It must be noted that, while Ptc is an integral membrane protein, ptcH84 enhancer-trap expression is nuclear. This is due to the localization of the lacZ enhancer-trap PtcH84 protein into the nucleus. This nuclear localization, and perhaps a greater stability of the lacZ enhancer-trap PtcH84 protein, seems to substantially enhance the level of detection of the Ptc expression domain. This enhancer-trap Ptc expression was however identical to that of wild-type Ptc expression as judged by whole-mount ptc-RNA in situ and staining of wild-type embryos with Ptc antibody (data not shown). This expression pattern indicates that Ptc is likely to be required in the neuroectoderm and/or in NB4-2.

The Patched function is required for the formation of NB4-2

It was fortunate that the enhancer-trap ptcH84 allele is also a phenotypic null allele of ptc (Hooper, 1995). Thus whether a partic-

![Fig. 1. Expression pattern of Ptc in the neuroectoderm and in neuroblasts. Stage 10 (~5.5 hours old) heterozygous ptcH84 embryos showing the expression of Ptc in the neuroectoderm (A) and in neuroblasts (B; lower right line drawing). This is a lacZ enhancer-trap allele of ptc and the expression pattern is assayed with β-gal antibody (the staining is therefore nuclear). Typically, row 2 and row 5 neuroblasts and the corresponding neuroectodermal cells have a higher level of Ptc than the remaining cells, especially when assayed by whole mount ptc-RNA in situ (not shown, see text). Anterior end is up, midline is marked by broken lines. Numbers along the midline (in B) indicate rows of neuroblasts. NB4-2 is marked by an arrowhead. The line drawing (top right) shows a partial map of S3 neuroblasts from row 4 to 7 and the expression pattern of a subset of proteins in these neuroblasts. The line drawing (bottom right) shows ptc-positive S3 neuroblasts. The numbers inside these neuroblasts indicate their identities. Note that the row 4 neuroblasts are also referred to as row 3 neuroblasts (see Skeath et al., 1995).](image-url)
The numbers inside these neuroblasts indicate their identities. Anterior end is up, midline is marked by broken lines. The numbers along the midline indicate rows of neuroblasts. (A) NB4-2 and other neuroblasts in this row are missing (open arrow). This failure in the delamination of NB4-2 is observed in 30% of the hemisegments. (B) A neuroblast in the position of NB4-2 is formed (arrowheads). The in the delamination of NB4-2 is observed in 30% of the hemisegments. (C) Stage not expressed in ‘NB4-2’ (open arrowhead). (D) Stage not expressed in ‘NB4-2’ (open arrowhead). The line drawings correspond to neuroblast maps in A and B, respectively. (E) A stage 10 (~6 hours old) wild-type embryo. Hkb is expressed in NB4-2 (Fig. 3A) and several other neuroblasts such as NB1-2, NB2-2, NB2-5, NB4-4 and NB5-4. In ptc mutants, as can be seen in Fig. 3C, Hkb-positive NB4-2, NB4-4 or NB5-4 were missing in these hemisegments. Note that the intervening space between the Hkb-expressing NB1-1 cells in the two hemisegments shown (Fig. 3C, large carets) is considerably reduced due to missing rows of neuroblasts. The failure in the formation of NB4-2 or the entire row 4 neuroblasts was also confirmed by double staining ptcH84 homozygous embryos with antibodies against β-gal and En (En marks the parasegmental boundary and is expressed in row 6 cells; see Fig. 1), β-gal and Wg (Wg is expressed in row 5 cells), by the position of the ectopic parasegmental boundary in ptc mutants (which forms between neuroblast rows 3 and 4) and by morphological landmarks such as tracheal pits. In the remaining 70% of the hemisegments (n=89), however, a neuroblast in the position of NB4-2 was clearly present (Fig. 2B, arrowheads; see below). In these hemisegments, several of the row 4 neuroblasts can also be seen (compare Fig. 2A and B), indicating that Ptc is not absolutely essential for the formation of these neuroblasts.

The Patched function is required for the specification of NB4-2 identity

The above results indicate that, in 30% of the hemisegments in ptc mutants, NB4-2 fails to delaminate from the neuroectoderm in ptc- embryos. Since NB4-2 segregates approximately one hour after the onset of gastrulation in mid to late stage 9 of embryogenesis (~4.5-4.75 hours old), stage 10-12 (~7-8.5 hours old) ptc- embryos stained with β-gal antibody were examined for the presence of NB4-2. The results from this experiment are shown in Fig. 2. As shown in Fig. 2A, in about 30% of ptc- hemisegments (the number of hemisegments examined, n=89), NB4-2 or the entire row of neuroblasts was missing (open arrow). This is also indicated by the expression pattern of NB4-2-specific enhancer-trap marker, Huckebeen 5953(Hkb) (Doe, 1992; Chu-LaGraff and Doe, 1993; Chu-LaGraff et al., 1995) in ptc- embryos. In wild type, Hkb is expressed in NB4-2 (Fig. 3A) and several other neuroblasts such as NB1-2, NB2-2, NB2-5, NB4-4 and NB5-4. In ptc mutants, as can be seen in Fig. 3C, Hkb-positive NB4-2, NB4-4 or NB5-4 were missing in these hemisegments. Note that the intervening space between the Hkb-expressing NB1-1 cells in the two hemisegments shown (Fig. 3C, large carets) is considerably reduced due to missing rows of neuroblasts. The failure in the formation of NB4-2 or the entire row 4 neuroblasts was also confirmed by double staining ptcH84 homozygous embryos with antibodies against β-gal and En (En marks the parasegmental boundary and is expressed in row 6 cells; see Fig. 1), β-gal and Wg (Wg is expressed in row 5 cells), by the position of the ectopic parasegmental boundary in ptc mutants (which forms between neuroblast rows 3 and 4) and by morphological landmarks such as tracheal pits. In the remaining 70% of the hemisegments (n=89), however, a neuroblast in the position of NB4-2 was clearly present (Fig. 2B, arrowheads; see below). In these hemisegments, several of the row 4 neuroblasts can also be seen (compare Fig. 2A and B), indicating that Ptc is not absolutely essential for the formation of these neuroblasts.

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Fig. 2. Mutation in ptc affects the formation of NB4-2. (A,B) stage ~10 (~5.5 hours) ptcH84 homozygous embryos stained with β-gal antibody. Anterior end is up, midline is marked by broken lines. The numbers along the midline indicate rows of neuroblasts. (A) NB4-2 and other neuroblasts in this row are missing (open arrow). This failure in the delamination of NB4-2 is observed in 30% of the hemisegments. (B) A neuroblast in the position of NB4-2 is formed (arrowheads). The line drawings correspond to neuroblast maps in A and B, respectively. The numbers inside these neuroblasts indicate their identities.

Fig. 3. Mutation in ptc affects the specification of NB4-2 identity. Anterior end is up, midline is marked by broken lines. The numbers along the midline indicate neuroblast rows. (A) Stage 10 (~6 hours old) wild-type embryo showing the expression of NB4-2 marker Hkb in NB4-2 (arrowhead), NBs 1-1 (large caret) and 2-2 (small caret) (see Chu-LaGraff et al., 1995). (B) Stage 10 (~6 hours old) ptcH84/ptcH84 embryo. Hkb is not expressed in ‘NB4-2’ (open arrowhead). (C) Stage 10 (~6.5 hours old) ptcH84/embryo. The hemisegments here are missing NB4-2 (and several other NBs as well). The Hkb-positive NB1-1 is marked with large carets. Note that the intervening space between the two NB1-1 cells (large carets) in the consecutive hemisegments in C is considerably reduced due to missing rows of neuroblasts. (D,E) eve staining of 13 hour (stage 14) wild-type and ptc embryos. In wild type (D), the RP2 neuron is marked by an arrowhead. Curved arrow indicates EL neurons. Open arrowheads in the ptc mutant (E) indicate approximate positions of missing RP2 neurons. In ptc mutants, the EL neurons are also missing (open arrow in E), however, this defect is only partially penetrant. (F,G) mAb 22C10 staining of 13 hour (stage 14) wild-type and ptc- embryos, respectively. 22C10 is a monoclonal antibody that stains mature RP2 neurons (arrowhead in F). In ptc mutants, mature RP2 neurons are missing (open arrowhead).
Therefore, in 30% of the hemisegments, the loss of RP2 neurons must be due to this failure in the formation of NB4-2. In the remaining 70% of the hemisegments (n=89), a neuroblast in the position of NB4-2 was present (Fig. 2B, arrowheads). However, it was possible that the identity of this ‘NB4-2’ in these ptc− hemisegments was not properly specified and as a consequence the RP2 neurons were missing from these hemisegments. To determine whether this is indeed the case, ptc homozygotes were examined for the expression of NB4-2 marker, Hkb. In ptc mutants, either a Hkb-positive NB4-2 is not formed (Fig. 3C; see also Fig. 2A) or when a neuroblast is formed in the position of NB4-2 (as in 70% of the hemisegments; see also Fig. 2B), it is negative for Hkb (Fig. 3B, open arrowhead). The absence of Hkb expression in ‘NB4-2’ of ptc mutants indicates that NB4-2 identity was not conferred on this neuroblast. Consistent with these results, Eve-positive GMC-1, its progeny RP2/sib or a mature RP2 neuron was not observed in ptc homozygotes (Fig. 3E, open arrowheads; see also Patel et al., 1989). Eve staining of ~13 hour old (stage 14) ptc− embryos indicates that, although partially penetrant, a group of 8-10 cells called EL neurons (Fig. 3E, curved arrow) are also missing in ptc− embryos (Fig. 3E, empty arrow; see also Patel et al., 1989). Since Ptc expression is mostly restricted to the neuroectoderm and neuroblasts, it is likely that the loss of EL neurons in ptc− embryos is due to a failure in the formation and/or specification of parent neuroblasts of EL neurons. While the Eve staining of ptc mutants indicates that Eve-positive mature RP2 neurons are missing in ptc− embryos, in those ptc− hemisegments where ‘NB4-2’ is formed, this neuroblast could generate a neuron that occupies the location of an RP2 neuron within the anterior commissure. For instance, loss

![Image](https://example.com/image1.png)

Fig. 4. The duplication of the RP2 lineage in gsb mutants is due to NB5-3 changing to NB4-2 identity: (A) Wild-type stage 10 (~6 hours old) embryo stained for the expression of NB4-2 marker Hkb (arrowhead). (B) Stage 10 (~6 hours old) gsb mutant embryo. Both NB4-2 and NB5-3 (arrowheads) express Hkb. Anterior end is up. Midline is marked by broken lines. Numbers along the midline indicate rows of neuroblasts. Numbers inside neuroblasts in the line drawings indicate neuroblast identities. (C,D) Wild type embryos and (E,F) gsb mutant embryos stained for eve. In the NB4-2→GMC-1→RP2/sib lineage, eve begins to be expressed in GMC-1 (C, thick arrows) but not in NB4-2. eve continues to be expressed as GMC-1 divides to give an RP2 (C, arrowhead) and a sib (thin arrow). Ultimately, eve disappears from sib but RP2 maintains eve (arrowhead in D). Note that development of this and other lineages are slightly asynchronous in different hemisegments of an embryo. (E) A ~7.5 hour (stage 11) gsb− embryo. The hemisegment on the left has three cells, GMC-1 (thick arrow), RP2 and sib (arrowhead and thin arrow). The hemisegment on the right has four cells. Here, both GMC-1 cells have divided to give rise to two RP2s (arrowheads) and two sibs (thin arrows). (F) A 13 hour (stage 14) gsb− embryo showing the duplication of the RP2 neurons (arrowheads). In the gsb mutant, aCC/pCC neurons are also duplicated (large and small carets), however, expressivity of this phenotype is partial. Furthermore, two other eve-positive lineages, Us and CQs (out of focus in these panels) are missing in gsb mutants (see Patel et al., 1989). (G) Wild type and (H) gsb mutant ~13 hour (stage 14) embryos stained with mAb 22C10. In wild type (G), a single RP2 neuron is present in a hemisegment (arrowhead), while in the gsb mutant (H), two RP2s can be observed in a hemisegment (arrowheads). Large carets indicate aCC neurons. Anterior end is up. Midline is marked by broken lines.
As shown in Fig. 3G, in axons, one of which is the RP2 neuron (Fig. 3F, arrowhead). Stains cell membrane of a number of neuronal bodies and their is a monoclonal antibody raised against a membrane protein and stained with an antibody, mAb 22C10 (Fujita et al., 1982). This approximate positions of missing RP2/sib cells in mutants. (B,C) indicated by arrowheads. Open arrowheads (B-E) indicate antibody in these panels. (A) Wild-type embryo. RP2/sib cells are dependent. ~8 hour (late stage 11) old embryos are stained with an antibody, Ptc which is expressed in neuroblast rows 5 and 6, and NB7-1 of row 7 and their axonal path finding is often affected in such a way that it sends out the axonal trajectory in the wrong direction (Doe et al., 1988). Therefore, to determine whether a neuron is present in the position of RP2 in ptc- embryos, ptc homozygotes were stained with an antibody, mAb 22C10 (Fujita et al., 1982). This is a monoclonal antibody raised against a membrane protein and stains cell membrane of a number of neuronal bodies and their axons, one of which is the RP2 neuron (Fig. 3F, arrowhead). As shown in Fig. 3G, in ptc- embryos, a 22C10-positive neuron in the location of the RP2 was not observed (the approximate position is marked with an open arrowhead). These results indicate that Ptc is required not only for the formation but also for the specification of NB4-2 identity, and loss of RP2 neurons in ptc mutants is due to failure in the formation as well as in the specification of the precursor neuroblast, NB4-2.

**Loss of Gooseberry transforms NB5-3 to NB4-2 thus duplicating the RP2 lineage**

By contrast to the loss of RP2 neurons in ptc mutants, in gsb mutants, the RP2 lineage is duplicated (Patel et al., 1989). Gsb is expressed in neuroblast rows 5 and 6, and NB7-1 of row 7 and their precursor neuroectoderm (Gutjahr et al., 1993; see also Fig. 6E,F). In gsb+, it is likely that one of these neuroblasts behaves as NB4-2. Indeed, a recent study suggests that in gsb mutants NB5-3 is transformed to NB4-2 (Skeath et al., 1995). This is illustrated in Fig. 4. As shown in Fig. 4B, in about 45% of the gsb- hemisegments (n=79), in addition to NB4-2 (upper arrowhead), NB5-3 also expresses the NB4-2 marker Hkb (lower arrowhead). This transformation of NB5-3 to NB4-2 in gsb mutants is also evident from the expression of Runt. In wild type, Runt is expressed in NB3-2 and NB5-3 but is not expressed in NB4-2 (see Chu-LaGraff and Doe, 1993). In gsb mutants, ‘NB5-3’ was negative for Runt (data not shown).

The transformation of NB5-3 to NB4-2 is also indicated by the dynamics of Eve staining in gsb mutants. Eve is expressed in the NB4-2→GMC-1→RP2/sib lineage beginning with GMC-1 (Fig. 4C, thick arrow) and continues to be expressed in newly formed RP2 and its sibling cell (Fig. 4C, thick and thin arrows). Soon after, sib loses Eve expression, but the RP2 neuron continues to express Eve (Fig. 4D, arrowhead; see Bhat and Schedl, 1994; Bhat et al., 1995). Thus Eve can be used to monitor the development of this lineage beginning with GMC-1. Since NB5-3 is formed during stage 8 (~4.5-4.75 hours old) of embryogenesis and NB4-2 is formed ~35 minutes later in stage 9 (~4.5-4.75 hours old), in gsb mutants, this temporal difference is expected to generate three Eve-positive cells in a hemisegment, a GMC-1 from NB4-2, and an RP2 and a sib from ‘NB5-3’, at some point during the development of this lineage. Indeed, as shown in Fig. 4E (lower left hemisegment) three Eve-positive cells – a GMC-1 (thick arrow), an RP2 (arrowhead) and a sib (thin arrows) – were observed in a ~7.5 hour (stage 11) embryo. Subsequently, the GMC-1 of NB4-2 should also divide to give rise to an RP2 and a sib, and thus four Eve-positive cells (two RP2s and two sibs) should also be observed in gsb- hemisegments. As shown in the upper right hemisegment in Fig. 4E, four Eve-positive cells were indeed observed in a gsb- hemisegment (see also Bhat and Schedl, 1994; Bhat et al., 1995). Since the development of the RP2 lineage (and other CNS lineages) is slightly asynchronous between hemisegments, three and four cells can be observed in a ~7.5 hour (stage 11) embryo (c.f., Fig. 4E). In ~13 hour (stage 14) gsb- embryos, two RP2 neurons instead of one were observed in ~50% of the hemisegments (n=350; Fig. 4F, arrowheads). Furthermore, with 22C10 staining, these duplicated RP2 neurons and their axonal trajectories can be seen in the location of RP2 within the anterior commissure (Fig. 4H, arrowheads). The absence of RP2 lineage duplication in the remaining 50% of the hemisegments (n=110) in gsb mutants is due to a failure in the formation of NB5-3 (data not shown; Skeath et al., 1995). These results indicate that, in gsb mutants, NB5-3 executes genetic programs characteristic of NB4-2 and produces neurons normally generated by NB4-2.

In gsb mutants, the Eve-positive aCC/pCC neurons are also duplicated (Fig. 4F, carets), although this phenotype is partially penetrant. This duplication of aCC/pCC neurons in gsb mutants is likely due to a second neuroblast assuming the identity of NB1-1, the parent neuroblast of aCC/pCC (although other possibilities such as a second GMC cell assuming the identity of GMC-1 of the aCC/pCC lineage, do exist). In addition, Eve staining of gsb- embryos also indicates that the Eve-positive neurons, CQs and Us, are also missing in these embryos (see also Patel et al., 1989). It seems most likely that the absence of these Eve-positive neurons in gsb mutants is due to a mis-specification and/or a failure in the formation of parent neuroblasts.

**The autonomous Wg signal is responsible for conferring NB4-2 identity to NB5-3 in gooseberry mutants**

In wild type, Wg is expressed in row 5 neuroblasts and their precursor neuroectoderm and this paracrine Wg is required for
the specification of NB4-2 identity (Chu-LaGraff and Doe, 1993). In gsb mutants, although NB5-3 is transformed into NB4-2 (this report; Skeath et al., 1995), this ‘NB5-3’ and the neuroectoderm from which NB5-3 delaminates still express Wg (data not shown). Therefore, I next determined whether the transformation of NB5-3 to NB4-2 in gsb mutants is dependent on an autocrine Wg signal. This was done by examining the RP2 lineage in wg;gsb double mutants. As shown in Fig. 5C, Eve staining shows that wg;gsb double mutants lack Eve-positive RP2 lineage just as in wg single mutants (Fig. 5B), indicating that Wg is required for NB5-3 (and NB4-2) to assume NB4-2 identity in gsb mutants. This extends previous observations (Skeath et al., 1995) and shows that the transformation of NB5-3 to NB4-2 in gsb mutants is due to the autocrine Wg signal specifying NB4-2 identity to this row 5 neuroblast in the absence of Gsb. Thus Gsb normally represses the ability of cells to respond to the Wg signal. Finally, the RP2 lineage was also missing in wg;ptc double mutants as in wg or ptc single mutants (Fig. 5D,E).

**patched and gooseberry interact during the specification of NB4-2 identity**

Analysis of genetic epistasis of mutants that have opposite phenotypes is often very informative in revealing their specific and interactive roles in determining cell fates (Siegfried et al., 1994; Noordermeer et al., 1994). Since ptc and gsb mutants had opposing phenotypes in the RP2 lineage (loss of RP2 neurons versus gain of RP2 neurons), I examined the genetic epistasis of these two mutants by analyzing embryos lacking both of these gene products. If the transformation of NB5-3 to NB4-2 in gsb mutants is still Ptc-dependent, the ptc phenotype is expected to be epistatic to the gsb phenotype and a loss of RP2 neurons will be observed. On the contrary, if Ptc is dispensable for the specification of the RP2 lineage as long as these embryos also lacked Gsb, a gsb phenotype is expected to be epistatic and a restoration of the RP2 lineage should be observed. Consistent with the latter possibility, in 70% of the hemisegments in ptc;gsb double mutants, the specification of the RP2 lineage was restored. In these double mutants, however, since NB5-3 is still transformed into NB4-2, the RP2 lineage was duplicated. These results are illustrated in Fig. 6A-D by Eve and 22C10 staining. In ~8.0 hour (late stage 11) gsb;ptc double mutant embryos (Fig. 6A), 36% of the hemisegments (n=110) had a newly formed RP2 and a sib, and about 35% of the hemisegments had either a GMC-1, a newly formed RP2 and a sib (three cells, not shown) or two pairs of newly formed RP2 and sib cells (four cells). In older ~13 hour (stage 14) embryos, hemisegments with one or two RP2s were observed (Fig. 6C). That these Eve-positive cells in the double mutant differentiate into mature RP2 neurons is also evident by the 22C10-staining pattern. As shown in Fig. 6D, a mature RP2 neuron with its axonal trajectory was observed in the ptc;gsb double mutant (arrowhead). Furthermore, in the double mutant, both NB4-2 and ‘NB5-3’ had Hkb expression and ‘NB5-3’ was Runt negative as in the gsb single mutant (data not shown). These results show that a loss of function for Gsb can suppress loss of NB4-2 identity in the ptc mutant.

Next, whether the rescue of the RP2 lineage in ptc;gsb double mutants is dependent on Wg was determined. This was done by examining the specification of NB4-2 identity in ptc gsb;wg triple mutants. In these triple mutant embryos, the NB4-2 identity was not specified and, as a consequence, the RP2 neurons were missing. These results indicate that specifi-
carnation of NB4-2 identity and thus the rescue of the RP2 lineage in ptc;gsb double mutants is dependent on Wg signal.

Although a loss of gsb activity from ptc embryos restores NB4-2 identity and thus the RP2 lineage, a similar suppression of the loss of EL neurons in ptc mutants was not observed in ptc;gsb double mutants. The EL neurons were still missing in ptc;gsb double mutants as in ptc single mutants. Similarly, the duplication of the aCC/pCC neurons or missing CQs and Us in gsb single mutants is not suppressed in the ptc;gsb double mutant. These results indicate that the interaction of ptc and gsb is specific to the RP2 lineage (among Eve-positive lineages) and not all lineages respond as the RP2 lineage to the simultaneous elimination of Ptc and Gsb proteins.

**patched and gooseberry do not interact with each other during the delamination of NB4-2**

The above results illustrate that, in ptc;gsb double mutants, the RP2 lineage is restored in ~70% of the hemisegments. However, as can be seen in Fig. 6B and C, in the remaining ~30% of the hemisegments (n=110) of ptc;gsb double mutants, the RP2/sib cells are still missing as in ptc single mutants (open arrowheads).

This percentage of hemisegments missing the RP2/sib cells in ptc;gsb double mutants is the same as that of the hemisegments in ptc single mutants missing NB4-2. Therefore, I conclude that elimination of Gsb from ptc embryos does not suppress failure in the delamination of NB4-2 due to the loss of ptc activity. These results indicate that ptc and gsb interact with each other only during the specification of NB4-2 identity but not during its delamination from the neuroectoderm.

**The Patched signaling pathway directly represses the Gooseberry expression in row 4 neuroblasts and their precursor neuroectoderm**

The analysis of ptc;gsb double mutants and ptc gsb;wg triple mutants indicate that Ptc represses expression of Gsb in NB4-2 and its precursor neuroectoderm (as well as in the other row 4 cells) and this repression is essential for the specification of NB4-2 identity by Wg. The Ptc signaling pathway could directly...
inhibit Gsb expression in row 4 cells alternatively, Ptc could block a Gsb-induced paracrine signal originating in row 5 cells from functioning in NB4-2 and/or its precursor neuroectoderm. To distinguish between these possibilities, ptc\(^{-}\) embryos were double stained with antibodies against Gsb (red) and En (purple). In wild type (Fig. 6E,F), Gsb is expressed in neuroblast rows 5 and 6, and NB7-1 of row 7 (Fig. 6F) and their precursor neuroectoderm (Fig. 6E). En is expressed in neuroblast rows 6 and 7, and NB1-1 of row 1 and their precursor neuroectoderm (Fig. 6E,F). In ptc mutants, as shown in Fig. 6G,H, while the expression of En remains the same as in wild type, the expression of Gsb was anteriorly expanded into row 4 neuroblasts (Fig. 6H) and their precursor neuroectoderm (Fig. 6G). This indicates that the Ptc signaling pathway directly inhibits Gsb expression in these row 4 cells. These results also indicate that, in ptc mutants, row 4 neuroblasts are transformed into row 5 neuroblasts, which is consistent with the observation that these row 4 cells in ptc mutants also express another row 5 marker, Wg (see Bejsovec and Wieschaus, 1993; Schuske et al., 1994; my unpublished results).

Taken together with the result that NB4-2 specification can proceed normally in ptc mutants as long as these embryos also lacked Gsb, and that this specification is dependent on Wg, the above results indicate that the ultimate function of Ptc during the specification of NB4-2 identity is to repress Gsb expression in NB4-2 and the neuroectoderm from which this neuroblast delaminates. This repression of Gsb by Ptc in turn allows the specification of NB4-2 identity by Wg. Thus when Gsb is present in a cell, it restricts the ability of this cell to respond to the Wg signal. These conclusions are also consistent with the previous finding that ectopic expression of Gsb (Hs-gsb) during neurogenesis causes loss of RP2 neurons (Zhang et al., 1994; Li and Noll, 1994; Skeath et al., 1995; see below), presumably by inhibiting the ability of cells to respond to the Wg signal. Moreover, the observation that ectopic expression of Ptc at very high levels from an inducible promoter can repress Gsb expression (Schuske et al., 1994) (ectopic expression of Ptc also represses Wg expression, see Schuske et al., 1994), also supports these above conclusions.

**Ectopic expression of Gooseberry in the neuroectoderm suppresses the RP2 lineage duplication induced by ectopic expression of Wingless**

While the results described in this paper and by others (Zhang et al., 1994; Skeath et al., 1995) indicate that in gsb\(^{-}\) embryos row 5 neuroblasts are transformed into row 4 neuroblasts, these row 5 's' cells (both neuroblasts and neuroectodermal cells) still express Wg until after stage 11 (~8 hours old) of embryogenesis (well after the specification of NB4-2 identity). This indicates that, in gsb mutants, NB5-3 still retains at least one of the characteristics of wild-type NB5-3. However, the observation that this Wg-positive 'NB5-3' in gsb mutants gives rise to an Eve-positive and a 22C10-positive mature RP2 neuron (Fig. 4F,H, arrowheads) indicates that expression of Wg in this 'NB5-3' or its precursor neuroectoderm has no consequence on its specification as NB4-2. In the absence of Gsb, NB5-3 is able to respond to the autocrine Wg signal and transform itself into NB4-2.

To further test my conclusion that Gsb restricts reception of the Wg signal, I analyzed the effects of ectopic expression of Wg as well as Wg and Gsb together on the specification of NB4-2. First, I expressed Wg ectopically using the inducible hsp 70 promoter at various developmental stages during embryogenesis. Hand-picked embryos (to ensure the accuracy of developmental stages) from a transgenic line carrying a wild-type wg gene driven by the hsp70 promoter (Hs-wg; see Nordermeer et al., 1992) were shifted from the non-inducible 22°C to the inducible 37°C for 20 minutes at different times during embryogenesis. These embryos were then returned to 22°C to allow the development to proceed for a specified length of time (see below) and then stained with Eve antibody. As indicated in Fig. 7C-F, ectopic expression of Wg prior to or during the development of the NB4-2\(\rightarrow\)GMC-1\(\rightarrow\)RP2/sib lineage did not result in missing RP2 neurons either in wild-type or in gsb mutant background. Instead, in wild type, a brief heat pulse ~25 minutes prior to the delamination of first wave of neuroblasts (S1 neuroblasts) induced duplication of the RP2 lineage in as many as 50% of the hemisegments. When stage 11 heat-shocked embryos (~8 hours old) were examined with Eve, as shown in Fig. 7C, four cells, i.e. two RP2s (arrowheads) and two sibs (thin arrows) instead of one RP2 and one sib, were observed (in slightly earlier stage embryos, three cells, i.e. GMC-1, newly formed RP2 and sib were observed, indicating...
that one of the GMC-1 cells divides first, data not shown). When stage 14 embryos (~13 hours old) were examined, two RP2 neurons were observed instead of one (Fig. 7D). With 22C10 staining, both of these RP2 neurons are found to be located in close proximity to each other within the anterior комиссure and project their axons to the intersegmental nerve bundle (data not shown). These results also indicate that Wg-positive cells can correctly respond to the Wg signal.

The results from the temperature-shift experiments shown in Fig. 7G indicate that ectopic Wg in the neuroectoderm prior to the formation of S1 neuroblasts induces duplication of the RP2 lineage (see also Chu-LaGraff and Doe, 1993; see Discussion). Indeed, examination of the expression of NB4-2 marker, Hkb, in Hs-wg embryos indicate that NB3-2 (an S1 neuroblast) behaves as NB4-2 (an S2 neuroblast) in these heat-shocked embryos (data not shown).

A similar ubiquitous expression of Wg in the gsb− background results in the triplication of the RP2 lineage (Fig. 7E,F). In an ~8 hour (late stage 11) gsb;Hs-wg embryo (Fig. 7E), six cells, three newly formed RP2s (arrowheads) and three newly formed sibs (thin arrows), were observed in a hemisegment. Consistent with this, in older embryos (~13 hr; stage 14), three mature RP2 neurons, instead of one, were observed (Fig. 7F, arrowheads). These three RP2 neurons were originating from ‘NB3-2’, ‘NB4-2’ and ‘NB5-3’, illustrating that, with the above genetic combinations, two additional neuroblasts in a given hemisegment can be induced to assume NB4-2 identity.

I next ectopically expressed Gsb using the inducible hsp 70 promoter (Hs-gsb) in the neuroectoderm prior to the formation of NB4-2. Consistent with the observation that de-repression of Gsb in row 4 cells in ptc mutants prevents the reception of the Wg signal, this ectopic expression of Gsb (Hs-gsb) resulted in a loss of RP2 neurons (data not shown; see Zhang et al., 1994; Li and Noll, 1994). Since Hs-gsb and Hs-wg produced opposing phenotypes in the RP2 lineage, genetic epistasis between the two would be useful to further test my conclusion that Gsb represses the competence of cells to respond to the Wg signal. As shown in Fig. 7G, ectopic expression of Gsb and Wg simultaneously in the neuroectoderm (Hs-gsb;Hs-wg) resulted in a loss of RP2 neurons. This shows that the Hs-gsb phenotype is epistatic to the Hs-wg phenotype and confirm that Gsb suppresses the ability of cells to receive the Wg signal (see Discussion).

DISCUSSION

The results described in this paper (summarized in Fig. 8) provide several novel insights into the inter-relationships of ptc, wg and gsb genes during neurogenesis and show how an individual neuroblast identity in a field is established through cell-cell signaling. The analysis of ptc mutants indicate that the Ptc signaling pathway is required for the formation, as well as for the specification, of neuroblast identity. While Ptc is likely to modulate neuroblast formation by interacting with proneural and neurogenic genes, the mis-specification of NB4-2 (and possibly other neuroblasts in this row) in ptc mutants is due to the de-repression of Gsb in the neuroectoderm from which NB4-2 is delaminated (Fig. 6G,H). This de-repressed Gsb prevents the reception of the Wg signal necessary for NB4-2 specification. Thus when both the gene products are removed (as in ptc;gsb double mutants), the loss of NB4-2 identity in ptc mutants is rescued in a Wg-dependent manner. In gsb mutants, the duplication of the RP2 lineage is due to a second neuroblast, NB5-3, assuming NB4-2 identity (Skeath et al., 1995; this report). The results presented here extend this observation and indicate that the transformation of NB5-3 into NB4-2 in gsb mutants is due to the autocrine Wg specifying NB4-2 identity to NB5-3 in the absence of Gsb.

The results presented in this paper also indicate that several distinct Ptc-mediated signaling pathways operate during neurogenesis. For example, failure in the delamination of NB4-2 in ptc mutants occurs only in ~30% of the hemisegments. In the remaining 70% of the hemisegments, NB4-2 is delaminated, however, its identity is mis-specified (see below). While the mis-specification of NB4-2 in ptc mutants can be suppressed by eliminating Gsb from these embryos (ptc;gsb double mutants), failure in the delamination of this neuroblast in ptc mutants is not suppressed by the elimination of Gsb. Therefore, the formation of neuroblasts and the specification of neuroblasts must be mediated by distinct Ptc-mediated signaling pathways.

The fate of NB4-2 in the patched mutant

The results described here show that NB4-2 suffers two different fates in ptc mutants. First, in ~30% of the hemisegments, NB4-2 fails to delaminate from the neuroectoderm. Second, in the remaining 70% of the hemisegments, while this neuroblast is
formed, its identity is not specified as NB4-2. The absence of expression of NB4-2 marker, Hkb, in ptc mutants indicates that NB4-2 assumes a identity different from that of NB4-2. The staining of ptc+ embryos with Gsb antibody shows that this ‘NB4-2’ and its precursor neuroectoderm inappropriately expresses Gsb in these embryos (Fig. 6G,H). Since Gsb is normally expressed in neuroblast rows 5 and 6, and NB7-1 of row 7 and the precursor neuroectoderm, it was possible that ‘NB4-2’ in ptc mutants is transformed into one of these Gsb-positive neuroblasts. However, the expression pattern of En in ptc+ embryos, which is same as wild type (Fig. 6G,H), indicates that this ‘NB4-2’ in ptc mutants does not assume row 6 or row 7 neuroblast identities (En is a marker for neuroblast rows 6 and 7). These results suggest that, in ptc mutants, row 4 cells are transformed to row 5. This conclusion is also consistent with the observation that, in ptc mutants, another row 5 marker, Wg, is also expanded anteriorly into row 4 cells (see Bejsovec and Wieschaus, 1993; data not shown). It is therefore likely that ‘NB4-2’ in ptc mutants is transformed into a row 5 neuroblast. Indeed, in ~2% of the ptc- hemisegments (n=209), ‘NB4-2’ was found to express a NB5-3 marker, Runt. However, loss of function for ptc appears to affect the identity of NB5-3 itself as indicated by their lack of Runt expression in ~50% of the ptc- hemisegments (n=80)(data not shown). Therefore, it seems likely that, in ptc mutants, ‘NB4-2’ assumes perhaps a partial NB5-3 identity.

Evidently, the results presented in this paper indicate that, in ptc mutants, the re-specification, but not the formation, of NB4-2 as a NB5-3-like cell is due to the inability to receive the Wg signal by the neuroectodermal cells from which NB4-2 is delaminated. This suggests that, in wg mutants, NB4-2 is also respecified as NB5-3. However, a previous study indicated that, in wg mutants, NB4-2 is respecified as a NB3-2-like cell (Chu-LaGraff and Doe, 1993). This conclusion was reached based on the observation that the level of Runt in ‘NB4-2’ in wg+ was the same as that of NB3-2. While both NB3-2 and NB5-3 express Runt, the intensity of Runt staining in NB5-3 is slightly lower than NB3-2 (see Chu-LaGraff and Doe, 1993). The caveat, however, is that occasionally NB5-3 also expresses Runt at the same level as NB3-2 (my unpublished results), and therefore it is possible that ‘NB4-2’ in wg mutants is respecified as NB5-3 (as in ptc) and not as NB3-2. Consistent with this possibility, a NB3-2-specific enhancer element of runt was not active in ‘NB4-2’ in wg mutants (Gergen, personal communication).

Furthermore, in wg mutants, nearly 80% of the hemisegments were missing NB4-2 (Chu-LaGraff and Doe, 1993) as opposed to ~30% in ptc mutants (Fig. 2). This would indicate that the two proteins mediate NB4-2 formation by different pathways. Indeed, the result that ptc, gsb and wg do not interact during neuroblast formation the same way as during neuroblast identity specification (Fig. 6B,C) strongly argues in favor of this possibility.

The individual neuroblast identities are specified in the neuroectoderm prior to their delamination as neuroblasts

When do neuroblasts assume their individual identity during neurogenesis? The previous results of Chu-LaGraff and Doe (1993) obtained using a temperature-sensitive allele of wg indicate that Wg is required in the neuroectoderm for the specification of NB4-2 identity. The results presented in this paper (Fig. 7G) provide additional evidence that the identity of a neuroblast is specified prior to its delamination as neuroblast and this depends on a combinatorial genetic program executed in a temporally precise manner in the neuroectoderm. First, the transformation of NB3-2 to NB4-2 in Hs-wg embryos occurs only when Wg is ectopically expressed in the neuroectoderm ~25 minutes before the delamination of NB3-2. The triplication of the RP2 lineage in Hs-wg;gsb embryos also follows a similar time frame. Ectopic expression of Wg later in development (just as NB3-2 is formed or thereafter), however, does not transform this (or any other) neuroblast into NB4-2 in wild-type or gsb mutant background. Second, ectopic expression of Gsb in the neuroectoderm prior to NB4-2 delamination (K.B., unpublished results) causes a loss of RP2 neurons, indicating that interaction between Gsb and Wg in the neuroectoderm prevents Wg from conferring NB4-2 identity. The fact that both Wg and Gsb are required in the neuroectoderm and that Ptc activity is essential for the repression of Gsb in order to confer NB4-2 identity to a neuroblast argues that Ptc is also required for the specification of NB4-2 identity in the neuroectoderm. These results indicate that the neuroectodermal expression of a subset of segmentation genes is utilized for the specification of neuroblast identity (see also Patel et al., 1989).

Responsiveness to the Wingless signal is controlled by the sequential negative regulation, patched→gooseberry→Wingless-receptor during NB4-2 specification

The lack of autocrine specification of NB4-2 in Wg-expressing row 5 cells suggests that the response to the Wg signal is spatially restricted, presumably through the activity of the Wg-receptor. The results described in this paper demonstrate that Gsb is one of the proteins controlling this response to the Wg signal. The results show that Wg can confer NB4-2 identity only in the absence of Gsb. Thus, in order to allow the reception of the Wg signal by row 4 cells, Gsb must be repressed in these row 4 cells. The results show that Ptc mediates such a repression of Gsb in these cells, allowing the reception of the Wg signal (see Fig. 9). In the absence of Ptc, Gsb is de-repressed in ‘NB4-2’ and its precursor neuroectoderm, which then blocks the reception of the Wg signal. When both the gene products are removed as in ptc;gsb double mutants, the cell is able to receive the Wg signal and assume the correct identity. In row 5, however, the cells must be prevented from receiving the autocrine Wg signal in order for these row 5 cells to assume identities different from that of row 4. To accomplish this, Gsb is uncoupled from Ptc regulation in these Wg-positive row 5 cells and Gsb (as well as Ptc) is expressed in these cells. Thus, the competence of these cells to receive the autocrine Wg signal is suppressed. When Gsb activity is absent, as in gsb mutants, these Wg-positive row 5 cells are able to receive the Wg signal and, as a consequence, their identity is transformed to that of row 4 cells. That Gsb can repress the ability of cells to respond to the Wg signal (either autocrine or paracrine) is further confirmed by the result that ectopic Gsb can suppress the duplication of the RP2 lineage induced by ectopic Wg (Fig. 8G).

How does Gsb block the ability of cells to receive Wg signal in row 5 cells in wild type or row 4 cells in ptc mutants? Since Gsb is a DNA-binding protein, one possibility is that Gsb represses the expression of a receptor for Wg. Alternatively, a
Gsb-induced activity somehow is interfering with the Wg signaling pathway. Based on the results presented in this paper, I propose that the responsiveness to the Wg signal is controlled by sequential negative regulation, ptc→gsb→Wg-receptor.

While ptc is required to repress gsb expression in row 4 cells, my results show that this regulatory interaction between ptc and gsb is uncoupled in row 5 cells, where both ptc and gsb are expressed. An unknown factor, which is absent in row 4 cells, must be blocking the Ptc signaling pathway from repressing gsb in these row 5 cells (see Fig. 9). The signaling molecule Hh is believed to interact with Ptc to prevent it from repressing wg in these Wg-expressing row 5 cells (see Klingensmith and Nusse, 1994). However, this Hh and Ptc interaction is not involved in blocking the repression of gsb by Ptc in row 5 cells since loss of hh does not lead to loss of Gsb expression from row 5 cells (K. B., unpublished results). Recently, I have identified a mutation in which Ptc is able to repress Gsb in row 5 cells (K. B., unpublished data). These results indicate that the ability to express gsb in row 5 cells, in spite of the presence of ptc, enables these cells to assume row 5 rather than row 4 identity. These results also illustrate the differing strategies employed by cells to communicate with each other in order to specify individual cell identities during development.

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