Segment polarity gene interactions modulate epidermal patterning in 
Drosophila embryos

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SUMMARY

Each segment of a Drosophila larva shows a precisely organized pattern of cuticular structures, indicating diverse cellular identities in the underlying epidermis. Mutations in the segment polarity genes alter the cuticle pattern secreted by the epidermal cells; these mutant patterns provide clues about the role that each gene product plays in the development of wild-type epidermal pattern. We have analyzed embryos that are multiply mutant for five key patterning genes: wingless, patched, engrailed, naked and hedgehog. Our results indicate that wild-type activity of these five segment polarity genes can account for most of the ventral pattern elements and that their gene products interact extensively to specify the diverse cellular identities within the epidermis. Two pattern elements can be correlated with individual gene action: wingless is required for formation of naked cuticle and engrailed is required for formation of the first row of denticles in each abdominal denticle belt. The remaining cell types can be produced by different combinations of the five gene activities. wingless activity generates the diversity of cell types within the segment, but each specific cell identity depends on the activity of patched, engrailed, naked and hedgehog. These molecules modulate the distribution and interpretation of wingless signalling activity in the ventral epidermal cells and, in addition, each can contribute to pattern through a pathway independent of the wingless signalling pathway.

Key words: segment polarity, wingless, patched, engrailed, naked, hedgehog, Drosophila, epidermal patterning, pattern formation, gene interaction

INTRODUCTION

Wild-type Drosophila embryos secrete a segmentally repeating pattern of cuticular structures that indicates a high degree of positional information within each segment (Fig. 1A,B; Campos-Ortega and Hartenstein, 1985; Lohs-Schardin et al., 1979). The ventral surface of each abdominal segment is covered with a belt of denticles and an expanse of naked cuticle (Fig. 1A). A denticle belt consists of 6 rows of denticles, each with a slightly different morphology (Fig. 1B). A denticle therefore records the positional information of the underlying epidermal cell that secretes it, and provides an easily scored marker of the final patterning decisions made by that cell.

Mutational analysis has revealed a number of genes that are required to specify segmental pattern in the developing epidermis of the Drosophila embryo (Wieschaus et al., 1984; Jürgens et al., 1984; Nüsslein-Volhard et al., 1984). Recent work has shown that these gene products interact with each other to generate pattern, but the mechanisms that drive this patterning process are not well-understood. Severe patterning defects are observed in wingless (wg) mutants, indicating that wg plays a central role in generating the wild-type pattern. Many of the segment polarity genes are thought to encode components of the wg signalling pathway; these genes include gooseberry, fused, armadillo, dishevelled, porcupine, smooth and zeste-white 3 (shaggy) (reviewed in Peifer and Bejsovec, 1992). In this paper, we focus our attention on wingless itself and on the segment polarity genes that show unique cuticle phenotypes and novel regulatory effects on wg expression: patched (ptc), engrailed (en), naked (nkd) and hedgehog (hh).

Four of these five genes have been characterized at the molecular level and their expression patterns are known. Fig. 1C shows the relationship of wg, ptc, en and hh expression with the pattern elements within the segment. wg is expressed in a row of cells that underlies the middle of the naked cuticle expanse (Baker, 1987). en (DiNardo et al., 1985; Fjose et al., 1985; Kornberg et al., 1985) and hh (Mohler and Vani, 1992; Lee et al., 1992) are both expressed in the two rows of cells just posterior to the wg row: one row gives rise to naked cuticle, the other to the first row of denticles (Hama et al., 1990; Dougan and DiNardo, 1992). These adjacent wg and en/hh expression domains are activated at stage 5 by pair-rule gene activity and, in wild-type, are stably maintained until late stages of embryonic development (reviewed in Akam, 1987; Ingham, 1988). ptc is initially expressed in all cells of the segment but its tran-
scription is shut off rapidly in the en cells (Nakano et al., 1989; Hooper and Scott, 1989). Later in development its expression is modulated again to give lower expression in cells at the middle of its expression domain. nkd has not yet been characterized molecularly, but our genetic data (see below) suggest that it may be widely expressed within the segment.

Communication between the epidermal cells plays an important role in generating these patterns of segment polarity gene expression (reviewed in Peifer and Bejsovec, 1992). A signal from the wg-expressing cells is required to stabilize en expression in the adjacent row of cells; in the absence of wg activity, en expression decays by stage 9 (Martinez Arias et al., 1988; DiNardo et al., 1988; Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991). wg is a secreted molecule that is detected in neighboring cells on either side of the wg-expressing stripe (van den Heuvel et al., 1989; Gonzalez et al., 1991) and therefore is probably itself the signal. In addition, a signal from the en/hh-expressing cells is required to stabilize wg expression; in the absence of en activity, wg expression decays during stages 10 and 11 (Martinez Arias et al., 1988; Bejsovec and Martinez Arias, 1991). Since en encodes a DNA-binding protein (Desplan et al., 1985), the signal is probably a molecule whose expression is regulated by en, rather than en itself. hh has been proposed as a likely candidate because wg expression decays in hh mutants (Hidalgo and Ingham, 1990). en is required for maintaining hh expression, and hh encodes a putative transmembrane protein that may be secreted (Mohler and Vani, 1992; Lee et al., 1992).

These en and hh-expressing epidermal cells in the most posterior part of the segment secrete the first row of denticles in the wild-type denticle belt. The segment border lies between the first row of denticles, which are small and point anteriorly, and the second row of denticles, which are longer and point posteriorly. The second and third row are similar in appearance but second row denticles, particularly those near the ventral midline, tend to be thinner and less sharply hooked. Fourth row denticles are small and point anteriorly. Fifth row denticles are large and thick, and point posteriorly. Sixth row denticles are very small and point posteriorly. The remainder of the segment consists of naked cuticle. Although pattern elements, such as muscle attachment sites, exist within this naked cuticle, we have not examined these in detail and will consider naked cuticle as a single pattern element in this paper.

The complex array of denticle morphologies in rows 2 through 6 indicates a high degree of patterning information in the cells underlying them, but this information apparently is not conferred by differential expression of known segment polarity genes. All of the cells underlying rows 2 through 6 express only ptc. Therefore we have analyzed genetic interactions among the segment polarity genes to try to understand how their products generate the array of positional values that gives rise to the wild-type denticle pattern. We find that wg gene activity is required to generate the diverse denticle types and that ptc, en, nkd and hh gene actions modulate the specific type of denticle produced by a cell. Since wg is a secreted molecule that appears to form a graded distribution across the segment (Gonzalez et al., 1991; Bejsovec and Martinez Arias, 1991), we propose that diverse cell types can be specified in response to different threshold levels of wg within the cell. We demonstrate that ptc and nkd activities are important in controlling the distribution of wg activity within the segment and that ptc, en and hh affect the response of a cell to wg signal. In addition, ptc, en, nkd and hh can contribute to pattern by a pathway independent of the wg signal.

**MATERIALS AND METHODS**

**Genetic analysis**

Mutations used in this analysis either are known to be molecular null mutations (wgCX2 and enSFX31) or are the strongest alleles available (ptcIN108, nkdE89 and hhG51). Other alleles used for comparison were wgG22, ptcD44, ptcF85, enR57, enIM99, hhIN108 and hhL129. Results obtained with these other alleles were essentially the same as those with the strongest alleles listed above unless noted otherwise in the text.

Standard recombination techniques were used to construct the doubly and triply mutant strains for wg, ptc and en, which are on the second chromosome, and to construct the doubly mutant nkd hh third chromosome. These strains were verified by complementation testing and were balanced with standard balancer chromosomes: CyO for the second and TM3 for the third. In examining multiply mutant phenotypes involving both the second and third chromosomes, we found it impossible to use doubly balanced strains. These strains produce variable, non-specific pattern defects that confound the analysis of both antibody staining and cuticle pattern. Therefore we chose to examine unlinked mutant combinations by directly crossing the singly balanced strains and mating the non-balancer progeny to each other. These outcrossed flies are very robust and their progeny rarely show non-specific defects. Because recombination can occur in the female germ line, all possible recombination events must be considered when scoring the progeny of females heterozygous for doubly or triply mutant chromosomes. The outcomes of these calculations are presented in condensed form in Table 1. Although outcrossing precludes the use of marked balancer chromosomes, we can assess cuticle patterns and antibody stainings accurately for all multiply mutant combinations and can assign phenotypes with statistical significance well above the required 5% level. In addition, recombination has revealed interesting heterozygous effects for some of the mutant combinations. These heterozygous phenotypes were verified by

![Fig. 1](image-url) **Fig. 1.** Cuticle patterns secreted by wild-type, singly and doubly mutant embryos. (A) Ventral surface of a wild-type first instar larva, showing the 8 abdominal denticle belts. In this and all subsequent pictures, anterior of the embryo is to the left. Scale bar is 100 μm. (B) Higher magnification of denticle belts in abdominal segments 5 and 6. Note that each of the 6 rows of denticles in each belt has a characteristic size and shape denticle. (C) Schematic sagittal view of the epidermal cells within a segment, showing the expression domains of wg (W), en (E), ptc (P) and hh (H). The first row of denticles is secreted by cells that express en and hh at the posterior of the preceding segment. (D) Cuticle patterns secreted by wgCX2 (W), ptcIN108 (P), enSFX31 (E), nkdE89 (N) and hhG51 (H) single mutants (diagonal row) and each possible pairwise combination of the five mutations. Magnification is the same as that of B. See Table 1 for assessment of denticle type identities and statistical significance of unlinked phenotypes. The wg; hh and en; hh double mutants produce uniform lawns of denticles but these do not photograph well because the cuticle is very poorly differentiated.
independent crosses to construct the relevant heterozygotes (data not shown). In some cases, these heterozygous effects change the expected ratio of progeny classes used to calculate the chi-square values presented in Table 1. The complete data tables are available from the authors upon request.

A number of unlinked mutant combination phenotypes are indis-

Fig. 1
### Table 1. Summary of single and multiple mutant phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dentine type</th>
<th>Ventral denticle pattern</th>
<th>wg distribution</th>
<th>en distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>N, 1 - 6</td>
<td>23456NNNNN1</td>
<td>OOOOOOOOO</td>
<td>OOOOOOOOOOOOO</td>
</tr>
<tr>
<td>W</td>
<td>5</td>
<td>lawn with segmental polarity reversals</td>
<td>decays at stage 10</td>
<td>decays at stage 10</td>
</tr>
<tr>
<td>P</td>
<td>N, 1, 2</td>
<td>22221NNNN1</td>
<td>OOOOOOOOO</td>
<td>OOOOOOOOOOOOO</td>
</tr>
<tr>
<td>E</td>
<td>N, 2 - 6</td>
<td>lawn with naked patches in alternate segments</td>
<td>decays at stage 10</td>
<td>n.d.</td>
</tr>
<tr>
<td>N</td>
<td>N, 1 - 6</td>
<td>belts missing in alternate segments</td>
<td>OOOOOOOOOOO</td>
<td>n.d.</td>
</tr>
<tr>
<td>H</td>
<td>5</td>
<td>disorganized lawn with central whorls</td>
<td>decays at early stage 9</td>
<td>persists in ventral and dorso lateral spots</td>
</tr>
<tr>
<td>WP</td>
<td>3</td>
<td>segmental lateral whorls</td>
<td>like W</td>
<td>like W</td>
</tr>
<tr>
<td>WE</td>
<td>5</td>
<td>lawn with central whorls</td>
<td>like W</td>
<td>n.d.</td>
</tr>
<tr>
<td>WN*</td>
<td>5, i</td>
<td>weak W</td>
<td>like W</td>
<td>n.d.</td>
</tr>
<tr>
<td>WH*</td>
<td>5</td>
<td>(37/252, (\chi^2=2.46, 2) d.f.) severe H</td>
<td>(72/283, (\chi^2=0.74, 2) d.f.)</td>
<td>(87/385, (\chi^2=0.345, 2) d.f.)</td>
</tr>
<tr>
<td>PE</td>
<td>2</td>
<td>22222NNN</td>
<td>like P</td>
<td>n.d.</td>
</tr>
<tr>
<td>PN*</td>
<td>N</td>
<td>only naked cuticle (77/194, (\chi^2=3.07, 2) d.f.)</td>
<td>like P but edges not sharp (32/464, (\chi^2=1.48, 3) d.f.)</td>
<td>(21/615, (\chi^2=0.697, 3) d.f.)</td>
</tr>
<tr>
<td>PH*</td>
<td>N, 1, 3, 4</td>
<td>33334NNNNN1</td>
<td>like P but levels not high (30/550, (\chi^2=0.82, 3) d.f.)</td>
<td>like wild-type (CNS like P) (43/608, (\chi^2=0.056, 3) d.f.)</td>
</tr>
<tr>
<td>EN*</td>
<td>N</td>
<td>only naked cuticle (87/526, (\chi^2=6.24, 2) d.f.)</td>
<td>some decay at stage 11 (pair-rule modulation) (39/498, (\chi^2=4.68, 2) d.f.)</td>
<td>n.d.</td>
</tr>
<tr>
<td>EH*</td>
<td>5</td>
<td>severe H (44/355, (\chi^2=0.952, 2) d.f.)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>NH</td>
<td>N, 1, 5, 6, i</td>
<td>NNN55555561</td>
<td>n.d.</td>
<td>like N</td>
</tr>
<tr>
<td>WPE</td>
<td>2</td>
<td>unpatterned lawn</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>WPEN*</td>
<td>2</td>
<td>disorganized WP (48/625, (\chi^2=5.54, 5) d.f.)</td>
<td>like P (CNS like WP) (16/450, (\chi^2=0.200, 4) d.f.)</td>
<td>like W (CNS as wild) (132/575, (\chi^2=0.523, 3) d.f.)</td>
</tr>
<tr>
<td>WP*</td>
<td>3</td>
<td>like WP, but embryo larger (32/429, (\chi^2=7.71, 5) d.f.)</td>
<td>like W but levels not high (30/550, (\chi^2=0.82, 3) d.f.)</td>
<td>n.d.</td>
</tr>
<tr>
<td>WEN*</td>
<td>5</td>
<td>like WE (170/484, (\chi^2=1.80, 4) d.f.)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>WEH*</td>
<td>5</td>
<td>severe H (101/940, (\chi^2=1.55, 4) d.f.)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>PEN*</td>
<td>N</td>
<td>only naked cuticle (107/668, (\chi^2=7.75, 4) d.f.)</td>
<td>like P until stage 10, then alternate stripes decay (29/441, (\chi^2=11.3, 4) d.f.)</td>
<td>n.d.</td>
</tr>
<tr>
<td>PEH</td>
<td>N, 2 - 6</td>
<td>23334(45)3NNN</td>
<td>like P until stage 11, some belts fused in pairs (112/921, (\chi^2=3.64, 5) d.f.)</td>
<td>n.d.</td>
</tr>
<tr>
<td>WNH*</td>
<td>5, i</td>
<td>like NH with no naked belts (116/1277, (\chi^2=6.73, 4) d.f.)</td>
<td>like NH (CNS like WP) (14/320, (\chi^2=7.200, 4) d.f.)</td>
<td>like NH (CNS as well) (132/575, (\chi^2=0.200, 4) d.f.)</td>
</tr>
<tr>
<td>PNH*</td>
<td>N, 1, 5, 6, i</td>
<td>like NH (151/488, (\chi^2=1.05, 4) d.f.)</td>
<td>like NH (106/480, (\chi^2=2.71, 4) d.f.)</td>
<td>like NH (106/480, (\chi^2=2.71, 4) d.f.)</td>
</tr>
<tr>
<td>ENH*</td>
<td>N, 5, 6</td>
<td>naked at midline, belt edges fused laterally (31/295, (\chi^2=5.25, 5) d.f.)</td>
<td>like NH (131/437, (\chi^2=1.53, 4) d.f.)</td>
<td>n.d.</td>
</tr>
<tr>
<td>WPEN</td>
<td>2</td>
<td>like WPE (253/673, (\chi^2=0.49, 5) d.f.)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>WPEH</td>
<td>2</td>
<td>like WPE (465/1310, (\chi^2=2.46, 4) d.f.)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>WPNH*</td>
<td>3</td>
<td>like WP (414/1166, (\chi^2=6.39, 6) d.f.)</td>
<td>like WP (60/195, (\chi^2=0.529, 7) d.f.)</td>
<td>n.d.</td>
</tr>
<tr>
<td>WENH*</td>
<td>5</td>
<td>like WE (365/1076, (\chi^2=4.46, 6) d.f.)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>PENH*</td>
<td>N</td>
<td>only naked cuticle (88/765, (\chi^2=0.63, 5) d.f.)</td>
<td>broad segmental domains ventrally, uniform dorsally (14/320, (\chi^2=0.85, 6) d.f.)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Genotypes are designated as: \(W = wg\), \(E = en\), \(P = ptc\), \(N = nkd\), and \(H = hh\). Cuticle pattern elements are assessed in columns 2 and 3. ‘Dentine type’ classifies the morphology of denticles produced based on their similarity to denticles in wild-type rows 1 through 6. ‘N’ indicates naked cuticle, and ‘i’ indicates small, indeterminate denticle types that are not represented in the wild-type pattern (see text). Overall arrangement of these denticles is described as ‘ventral denticle pattern’. \(wg\) and \(en\) expression patterns are described in columns 4 and 5. Circles indicate the 10 rows of cells in a typical abdominal segment at late stage 10, with RNA distribution for \(wg\) and protein distribution for \(en\) schematically depicted. All verbal descriptions of \(wg\) expression refer to protein distribution based on antibody staining, \(wg\) expression in \(wg\) mutants was assessed using the mutant allele \(wg^{d11}\), which produces detectable protein. Genotypes that involve mutations on both the second and third chromosome are indicated by asterisks and statistical analyses of the phenotypes observed are presented as chi-square values. Cuticle pattern was scored in embryos that failed to hatch from an unbalanced cross (excludes wild-type siblings); antibody staining was scored in all embryos produced from an unbalanced cross (includes wild-type siblings). Statistical significance in most cases is near or greater than 90%.
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Distinguishable from one of their parental phenotypic classes. In these cases, the reader is referred to Table 1 for the statistical evidence for this assignment of phenotype. Unlinked mutant combinations that produce a novel phenotype are documented photographically.

**Cuticle preparation**

Embryos were collected on apple juice agar plates and aged for 24 hours to allow the wild-type siblings to hatch. Unhatched embryos were dechorionated in bleach and mounted in Hoyer’s medium mixed 1:1 with lactic acid as described by Wieschaus and Nüsslein-Volhard (1986). To ensure accurate representation of all progeny classes, embryos were not dissected free of their vitelline membranes. All unhatched embryos from a given cross were mounted in rows on a single slide and scored using a multichannel counter.

**Expression analysis**

Antibody staining was performed as described by DiNardo et al. (1985). RNA in situ hybridizations were performed as described by Tautz and Pfeifle (1989). Embryos were staged according to Campos-Ortega and Hartenstein (1985).

**RESULTS AND DISCUSSION**

Analysis of double, triple and quadruple mutant combinations of null or very strong alleles allows us to assess the extent to which an individual gene product contributes to patterning. Even in mutant combinations, specific cell identities can be recognized by the characteristic cuticular structure that a cell secretes (see Fig. 2). We classify a mutant cell type by the wild-type denticle type that it most closely resembles (Fig. 2A). Only in a few mutant combinations are denticles produced that do not fall into a recognizable wild-type category; we refer to these as indeterminate denticle types.

We find only two wild-type pattern elements that can be assigned to the activity of individual genes. The naked cuticle expanse depends absolutely on *wg* activity, and the cell identity that produces row-1-type denticles is specified by *en* gene activity, in conjunction with *wg* activity. Cell types giving rise to the other components of wild-type pattern are generated by an interplay of all five genes. In particular, *wg* activity is required to produce the diversity of cell types, while *ptc, en, nkd* and *hh* show genetic interactions that modulate this generation of diverse cell types.

**wingless specifies naked cuticle and denticle diversity**

The first row of Fig. 1D shows double mutant combinations lacking *wg*. *wg* mutants secrete cuticle with a single type of denticle, that characteristic of the wild-type row 5, arranged in a segmentally repeating pattern of polarity reversals (see also Baker, 1988). *wg* mutants, and all mutant combinations lacking *wg*, fail to make naked cuticle in the ventral portion of the segment, therefore *wg* activity is essential for specifying this component of the wild-type pattern.

![Fig. 2](image)

**Fig. 2.** Assignment of denticle type identities. (A) High-magnification view of half of a wild-type denticle belt, showing the denticle types characteristic of rows 1 through 6. Ventral midline is at top, ventrolateral edge is at bottom. Because denticle morphologies in rows 2 through 4 vary from ventral to ventrolateral regions, we confine our analysis to denticle types at or near the ventral midline. (B) *wg ptc en* (*WPE*) triple mutants secrete long thin denticles most similar to wild-type row 2 denticles at the ventral midline. (C) *wg ptc* (*WP*) double mutants secrete larger denticles, with thicker bases and sharper hooks. This morphology appears to be most similar to that of wild-type row 3 denticles. (D) *wg* (*W*) single mutants secrete large, curved denticles that appear slightly refractile due to their thickness. These are similar in shape and thickness to wild-type row 5 denticles.
ptc, en, nkd and hh alter the wg cuticle pattern in double mutant combinations with wg, indicating that all four gene products can contribute to the final pattern through processes that do not require wg activity. These processes affect the polarity and/or morphology of individual denticle types (see below). These wg double mutant combinations share with the wg single mutant a general uniformity of denticle type (with the possible exception of the wg:nkd double mutant, discussed below). The specific type of denticle produced depends on the action of other segment polarity genes, but wg gene activity is required for more than one type of denticle to be produced simultaneously. Therefore wg acts to generate the diversity of denticle types secreted by the wild-type embryo, perhaps by providing a graded distribution of signal.

**engrailed controls denticle orientation and affects denticle morphology**

In the wg en double mutant, the segmental polarity reversals typical of wg single mutants are not observed (Fig. 1D; see also Bejsovec and Martinez Arias, 1991). The effect of removing en is unexpected because en is expressed only briefly in wg mutants. en is activated in stripes of cells at stage 5 (roughly 3 hours of development) as it is in wild-type, but decays during stages 8 and 9 (Martinez Arias et al., 1988; DiNardo et al., 1988; Bejsovec and Martinez Arias, 1991). It is no longer detectable by 4.5 hours of development in wg mutants. Despite its short duration, this early en expression is sufficient to alter denticle polarity in a segmental manner when the cuticle is secreted at 16 hours of development.

In wg en mutants, circular whorls are located at the ventral midline with the denticles pointing outward. No whorls are visible in the wg ptc en triple mutant (Fig. 3B). Because the only genetic difference between these two embryos is the presence of wild-type ptc gene activity in the wg en double mutant, we infer that ptc gene activity is required for formation of the wg en ventral midline whorls. These whorls indicate loss of the information specifying that denticles be oriented perpendicular to the anteroposterior axis. ptc gene activity may normally contribute some of this information because ptc expression is repressed in the stripe of en-expressing cells (Hidalgo and Ingham, 1990). The ubiquitous ptc activity produced when en activity is absent (Hidalgo and Ingham, 1990) may result in the circular orientation of denticles. hh single mutants (Fig. 1D, 4F) show a pattern of ventral whorls similar to the wg en pattern; this may reflect a similar effect on ptc activity.

The wg ptc double mutant also alters the polarity reversals seen in wg single mutants; wg ptc mutants show a subtle segmental pattern of lateral whorls that depends on en gene activity (Fig. 1D, 3A; see also Hidalgo, 1991). In addition, ptc mutation affects the morphology of denticles produced. Rather than the large thick denticles typical of row 5 (Fig. 2D), smaller denticles more typical of row 3 are produced (Fig. 2C). This suggests that the cells have undergone a uniform shift to a more anterior cell fate. A further shift in fate is seen in the triple mutant wg ptc en (Fig. 2B). These triply mutant embryos secrete a uniform lawn of small, fine denticles typical of row 2. The difference in denticle morphology between wg ptc and wg ptc en mutants must result from en activity in the wg ptc mutants. As in the wg single mutant, en is transiently expressed between 3 and 4.5 hours of development and its expression is restricted to stripes (not shown). Because denticle identities throughout the ventral epidermis are affected in wg ptc versus wg ptc en mutants, the effects of early striped en activity must be detected by all cells of the segment. Therefore en activity appears to produce a signal that is communicated to other cells. This particular signal cannot be hh because the effect is still observed in the absence of hh. wg ptc; hh mutants produce row-3-type denticles (Fig. 3G), while wg ptc en; hh mutants produce row-2-type denticles (Table 1).

The en signalling may require nkd gene activity. The wg ptc; nkd triple mutant (Fig. 3C) uniformly secretes denticles that are more typical of row 2 than row 3. Thus wg ptc; nkd resembles the wg ptc en triple mutant (Fig. 3B) more than it does the wg ptc double (Fig. 3A). This suggests that nkd gene activity may be required for en activity to influence denticle morphology across the segment.

**wingless phenotype is suppressed by nkd and enhanced by hh**

wg embryos are substantially smaller than wild-type embryos. This difference in size is due in part to the smaller size of cells that underlie denticle belts versus naked cuticle (see Peifer and Bejsovec, 1992; Bate and Martinez Arias, 1991) and also because wg embryos experience considerably greater cell death than wild-type embryos (Perrimon and Mahowald, 1987; Klingensmith et al., 1989). nkd appears partially to suppress this cell death. The wg; nkd doubly mutant embryo is slightly larger than the wg single mutant, with a larger field of denticles that shows more pronounced segmental modulation of denticle orientation and deeper segmental indentation of the denticle belt lateral margin (Fig. 1D). This deeper indentation suggests that wg does not specify the naked cuticle at the lateral margin of wild-type denticle belts. There also appears to be an indeterminate denticle type specified in addition to the row-5-type denticles; thus this may be an exception to the rule that loss of wg activity always results in uniformity of denticle type.

The effect of nkd on the wg cuticle phenotype requires wild-type activity of ptc and en, but not hh. The suppression is not apparent in the wg ptc; nkd triple mutant (Fig. 3C) nor in the wg en; nkd triple mutant (Fig. 3D), both of which are as small as the wg single mutant. In contrast, wg; nkd hh triple mutants (Fig. 3M) are large and also show indeterminate denticle types similar to those seen in wg; nkd mutants. The wg; nkd suppression is not observed in wg; nkd double mutants that are also heterozygous for en (not shown). Therefore the gene dosage of en is crucial for altering the wg; nkd cuticle pattern. Since the decay of en expression in wg; nkd mutants is indistinguishable from that of wg single mutants (Table 1), the suppression is not mediated through stabilized en expression. The suppression depends on the same early transient expression of en that is responsible for the segmental denticle polarity reversals observed in wg single mutants. This effect may be enhanced in the absence of nkd activity to give the more exaggerated segmental modulation in the wg; nkd double mutant. Thus, the effect of transient en expression on polarity may be separate and
distinct from its effect on wg ptc denticle morphology, discussed above. The effect on polarity is independent of nkd gene activity, as it is still observed (and is, in fact, enhanced) in wg nkd double mutants, whereas the effect on denticle morphology appears to be dependent on nkd gene activity.

Fig. 3. Cuticle patterns of doubly and triply mutant embryos. Genotypes are designated as: W = wg\textsuperscript{CX4}, E = en\textsuperscript{SFX31}, P = ptc\textsuperscript{IN108}, N = nkd\textsuperscript{789} and H = hh\textsuperscript{GS1}. See Table 1 for assessment of denticle type identities and statistical significance of unbalanced phenotypes.
Loss of hh activity makes the wg mutant phenotype more severe. The wg; hh double mutant is smaller than wg or hh single mutants and the cuticle is poorly differentiated (Fig. 1D). This phenotype may result from increased cell death. In any case, the wg; nkd increase in embryo size is epistatic to the wg; hh decrease in embryo size. wg; nkd hh triple mutants (Fig. 3M) show suppression of both the wg and hh mutant phenotypes.

\textit{engrailed} specifies row-1-type denticles

The \textit{en} single mutant is able to produce all denticle types except row-1-type (Fig. 1D; see also Kornberg, 1981). These denticle types are mixed in a random, variable fashion and show no apparent anteroposterior organization. The naked cuticle between these belts is variable in size and is observed only in alternate (odd-numbered) segments. All mutant combinations lacking \textit{engrailed} fail to produce row-1-type denticles, demonstrating that \textit{en} activity is essential for producing this cell identity. In addition, this manifestation of cell identity requires exposure of the cells to wg signal. Under no circumstances are row-1-type denticles secreted by mutants lacking wg activity (Table 1).

The pattern of wg expression in \textit{en} single mutants can account for the pattern of naked cuticle produced in alternate segments. wg-expressing cells require a signal from the adjacent \textit{en/hh}-expressing cells in order to maintain their wg expression (Martinez Arias et al., 1988). In \textit{en} mutants, wg begins to decay in even-numbered segments at 5 hours (stage 10; Fig. 5K), then disappears from all segments after germ band retraction (Bejsovec and Martinez Arias, 1991). Experiments with a temperature-sensitive allele of wg have shown that any wg activity after 6 hours of development directs specification of naked cuticle (Baker, 1988; Bejsovec and Martinez Arias, 1991). Therefore, the persistence of wg expression in odd-numbered segments through germ band retraction (7-9 hours) is sufficient to specify naked cuticle, resulting in the patches of naked cuticle observed in \textit{en} mutants. The naked cuticle patches are clearly due to wg activity as they are absent in \textit{wg en} double mutants (Fig. 1D), but wg does more than just specify naked cuticle in \textit{en} mutants. In \textit{wg en} double mutants, only row-5-type denticles are produced, as opposed to the many denticle types present in \textit{en} single mutants. Thus wg must contribute to the specification of denticle types 2, 3, 4 and 6.
Fig. 5. wg expression in wild-type and mutant embryos. Wg protein distribution or wg RNA (C and M) are shown in abdominal segments 4 through 8 of extended germ band embryos. Embryos are oriented with anterior to the left and ventral side down, but due to curvature of the germ band, the ventral surface of abdominal segments 4 through 8 faces up with posterior to the left. Arrowhead in A marks the ventral midline at the posterior end of the germ band. All embryos are shown at the same magnification. Embryos in C and M appear slightly larger due to different fixation methods used in RNA in situ hybridizations. (A) Wg antibody staining in a wild-type stage 11 embryo shows that wg expression is restricted to a narrow stripe of cells across the ventral portion of each segment. A dorsal spot of expression in each segment is out of focus. (B) In hh mutants, wg expression is no longer detectable by stage 10. (C) In ptc mutants, the wg transcription domain expands in an anterior direction. (D) In ptc en double mutants, Wg protein and wg RNA (not shown) distribution is indistinguishable from ptc single mutants. (E) ptc; hh double mutants also show an expanded wg expression domain, but the level of expression appears to be lower than that in ptc or ptc en mutants. (F) In wg mutants, wg expression decays during stages 10 and 11. This wg mutant allele, wg\textsuperscript{IL114}, makes a protein that is stable but not functional. (G) wg ptc double mutants carrying the wg\textsuperscript{IL114} allele show decay of wg expression at a similar stage. No expansion of the wg expression domain is observed in these mutant embryos at any stage. (H) nkd mutants show a normal endogenous wg stripe and an ectopic wg stripe 3-4 cells anterior to the endogenous stripe. (I) The ptc; nkd double mutant shows anterior expansion of wg expression as in ptc single mutants, but ptc; nkd expression levels appear to be lower than in ptc mutants. (J) In nkd hh double mutants, most wg expression decays early, as it does in hh single mutants. (K) en mutants show late decay of wg expression. At stage 11, wg is still detected at high levels in odd-numbered stripes. (L) The en; nkd double mutant does not show decay of wg expression. Instead, the wg domain appears to be expanded in a posterior direction (compare distance between A7 and A8 stripes in K and L). (M) In ptc en; nkd triple mutants, wg RNA is distributed uniformly in the ventral and ventrolateral segmented epidermis. The dorsolateral and terminal regions do not express wg, indicating normal regulation of wg in these regions. (N) ptc en; hh triple mutants show expanded wg domains but expression decays in even-numbered stripes (as in en single mutants). Odd-numbered segments continue to express wg after germ band retraction (unlike en single mutants). (O) ptc en; nkd hh quadruple mutants show a segmental repression of wg expression in the ventral region. Dorsal regions show ubiquitous wg expression, as in ptc en; nkd mutants.
Cuticle pattern of indviduals indicates that however, both hh types denticles may mark a default cell identity in the absence of 5D) and specifies the expanses of naked cuticle, no denticle specification is either the (Mohler and Vani, 1992; Lee et al., 1992). However, the consequences of hh and en gene activity in embryonic patterning are very different. hh is not absolutely required for the specification of row-1-type denticles, as is en. Row-1-type denticles form in the absence of hh in ptc; hh and nkd double mutants (Fig. 3F,K). Furthermore, the en/hh double mutant appears to experience increased cell death and produces a cuticle that is more severely defective than is either the en or hh single mutant phenotype (Fig. 1D). This indicates that hh and en have at least some functions that do not overlap.

In hh single mutants, both wg and en expression decay as the germ band extends (Figs 5B, 6G). Small remnants of the en stripe persist until late in development, but wg expression is not detected after the germ band has finished extending. However, both wg and en must contribute to the hh cuticle pattern because both the wg; hh and the en; hh double mutant phenotypes are more severe than is the hh single mutant phenotype (Fig. 1D), and the wg en; hh triple phenotype is more severe than is either double mutant (Fig. 3E).

**patched contributes to the wg-mediated generation of cell type diversity**

The cuticle pattern of ptc single mutants shows a mirror-image duplication of the anterior portion of each denticle belt (Figs 1D, 4D; see also Nüsslein-Volhard and Wieschaus, 1980). Only denticles typical of rows 1 and 2 are secreted, separated by expanses of naked cuticle. This phenotype corresponds with anterior expansion of the wg-expression domain (Fig. 5C), and expression of an ectopic stripe of en in the cells underlying the posterior edge of the denticle belt (Fig. 6H; see also Martinez Arias et al., 1988; DiNardo et al., 1988).

In the ptc en double mutant, only row-2-type denticles are produced, separated by expanses of naked cuticle (Figs 1D, 3H). Therefore the en expression in ptc mutants specifies the row-1-type denticles in the anterior and posterior of the mutant denticle belt (see also DiNardo et al., 1988). The two en stripes may also contribute to the precise orientation of denticles in the ptc mutant belts, as that organized arrangement is not observed in the ptc en double mutant. Row-2-type denticles may mark a default cell identity in the absence of ptc and en. In ptc en double mutants, these denticles are arranged in belts interspersed with naked cuticle specified by wg activity. When wg activity is removed, in the wg ptc en triple mutant (Fig. 3B), the row-2-type denticles appear in a uniform lawn.

**ptc activity is required for wg to function in generating cell diversity.** Although wg is present in ptc en mutants (Fig. 5D) and specifies the expanses of naked cuticle, no denticle types other than row 2 are produced. The loss of denticle diversity is not an inherent feature of ptc mutation. Both ptc en; hh (Fig. 3I) and ptc; nkd hh (Fig. 3L) triple mutants produce several different denticle types. Therefore, eliminating hh gene function restores some denticle diversity in the absence of ptc activity. Since wild-type hh gene activity appears to inhibit the wg-mediated generation of cell type diversity, we propose that hh also plays a role in modulating the ability of cells to respond to wg signalling.

**patched and naked may affect wingless autoregulation**

Wingless activity is required to maintain its own expression (Bejsovec and Martinez Arias, 1991). In wg homozygotes that make detectable but non-functional protein, Wg protein is activated by pair-rule gene action at the onset of gastrulation (stage 8, 3 hours of development), but decays in all segments during the extended germ band stages 10 and 11 (5.5-7 hours; Fig. 5F). wg activity therefore has an autocatalytic effect that is essential for its own continued transcription. Since Wg protein is secreted, this autocatalytic effect may not be restricted to those cells initially expressing wg. Wg protein entering neighboring cells may have the potential to activate wg expression de novo in these cells. Based on our analysis of wg expression in embryos homozygous for ptc or nkd, we propose that the autocatalytic effect can extend 3 to 4 cells anterior or posterior to the wild-type wg-expressing domain.

In ptc mutant embryos, wg expression rapidly expands during stages 8 and 9, resulting in a wg transcription domain that usually extends 3 cells anterior to the initial wg stripe (Fig. 5C; see also Martinez Arias et al., 1988; DiNardo et al., 1988). This expansion requires functional wg activity. Using the wg allele, which produces detectable but non-functional gene product (Gonzalez et al., 1991), we have constructed a wg ptc doubly mutant strain. No expansion of the mutant wg expression domain is observed at stages 8 and 9 (Fig. 5G), when such expansion would be detected in the ptc single mutant. Furthermore, wg expression decays during the extended germ band stages, as it does in the wg single mutant (Fig. 5F). This indicates that wg activity is required for the expansion of its own expression domain in ptc mutants, as well as for maintenance of its own expression. Therefore, wild-type ptc activity blocks the autacatalytic effects of wg activity in the cells anterior to the wg transcription domain and thus prevents inappropriate wg expression in these cells. For instance, ptc may play a role in restricting Wg protein transport or uptake (see below) so that cells anterior to the endogenous wg stripe are not exposed to levels of secreted Wg protein that will trigger the autocalytic activity.

Inappropriate wg expression is also observed in nkd embryos. An ectopic stripe of wg gene product is detected in stage 10 (mid-extended germ band) embryos, in cells that would give rise to the posterior edge of the denticle belt (Fig. 5H; see also Martinez Arias et al., 1988). As in the ptc situation, this ectopic wg expression depends on wg activity as it is not observed in wg; nkd mutant embryos that produce detectable wg product (Table 1). The normal and ectopic wg stripes in nkd single mutants are separated by an expanded domain of en-expressing cells (Fig. 6B; see also Dougan and...
DiNardo, 1992). This en activity must block wg expression because in the absence of en activity, wg is expressed in these cells. In the en; nkd double mutant, wg protein (Fig. 5L) and RNA (not shown) extend 3 to 4 cells posterior to the endogenous wg stripe; this expanded domain may include the row of cells that would express wg ectopically.

Fig. 6. en expression in wild-type and mutant embryos. (A) At stage 11, stripes of 2 to 3 en-expressing cells are seen in each wild-type segment. (B) In nkd mutants, the en-expressing domain is expanded in a posterior direction: even-numbered en stripes are 4 to 5 cells wide, odd-numbered en stripes are 3 to 4 cells wide. (C) In ptc; nkd double mutants, the en-expressing domain is expanded one cell further posteriorly: even-numbered en stripes are 5 to 6 cells wide, odd-numbered en stripes are 4 to 5 cells wide. This is the widest extent of en expression we have observed in any mutant combination; the pair-rule modulation suggests that pair-rule gene input determines the domain of cells competent to express en. (D) In nkd hh double mutants, the en domain is 3 to 4 cells wide in all segments. This slight expansion does not show pair-rule modulation. (E) In wild-type embryos at stage 12, when the germ band has retracted, en stripes are one cell wide across the ventral region. (F) ptc; hh double mutants have an epidermal en staining pattern that is indistinguishable from wild-type. This embryo can be unambiguously identified as a ptc; hh double mutant because it shows a mutant en pattern in the central nervous system (out of focus at the ventral midline) and because an ectopic groove is often visible midway through the segment, similar to the groove that forms at the ectopic en stripe in ptc single mutants. (G) hh mutants show decay of en expression except for portions of the stripe in the ventral and ventrolateral regions. These cells stably maintain their en expression through germ band retraction. en expression in the central nervous system also is disrupted: the en-expressing cells at the ventral midline are not detected in hh mutants. (H) ptc mutants at stage 12 show a complete ectopic en stripe 3 cells posterior to the endogenous en stripe, as well as an increased number of en-expressing cells in the central nervous system (out of focus at the ventral midline, see also Patel et al., 1989). (I) In wg mutants, epidermal en expression is lost early in development. By stage 12, shown here, only discrete groups of cells in the central nervous system are still expressing en. (J) wg; hh mutants show complete decay of epidermal en expression, as seen in wg single mutants. This embryo can be unambiguously identified as a wg; hh double mutant because it shows the hh mutant effect in the central nervous system: loss of the en-expressing cells at the ventral midline. (K) wg ptc mutants show almost complete decay of epidermal en expression, as in the wg single mutant, although in some embryos a few epidermal en-expressing cells can be detected in the ventral portion of the stripe. The doubly mutant embryos also show a mutant effect on the central nervous system: the en-expressing cells form a continuous line (out of focus at the ventral midline). (L) wg ptc; nkd triple mutants show stable epidermal en expression in ventral and ventrolateral regions of the endogenous stripe, similar to that seen in hh mutants. These embryos still show the wg ptc mutant effect in the central nervous system.
in the nkd single mutant. Thus, as with ptc, wild-type nkd activity may restrict the potential for secreted wg product to activate its own expression in neighboring cells.

In the ptc; nkd triple mutant, wg is transcribed in all cells of the segment (Fig. 5M). Initially, the expression pattern resembles that of ptc mutants (Fig. 5C), with anterior expansion, but by stage 10 wg RNA is uniformly expressed throughout the segment. We conclude that regardless of their initial pair-rule input, all epidermal cells in the ventral portion of the segment can potentially express wg. In ptc and nkd mutant embryos, wg expression does not depend on input from the en-expressing cells for its maintenance. In ptc en (Fig. 5D; see also Tabata et al., 1992), en; nkd (Fig. 5L) and ptc en; nkd (Fig. 5M) mutant embryos, wg expression does not decay as it does in en mutants (Fig. 5K). Therefore in the wild-type situation, en-expressing cells may act to maintain wg expression in the neighboring stripe of cells by counteracting some property conferred by ptc and nkd gene activity. For example, a signal from these cells may override an inhibitory effect of ptc and nkd on wg autoregulation within the wg-expressing cells.

The naked phenotype results from inappropriate wg activity

The nkd single mutant phenotype shows essentially wild-type denticle belts that are variably eliminated and replaced with naked cuticle (Figs 1D, 4A; see also Nüsslein-Volhard and Wieschaus, 1980; Jürgens et al., 1984). This inappropriate specification of naked cuticle requires wg activity. In the wg; nkd double mutant, no naked cuticle is observed across the ventral region (Fig. 1D). Furthermore, in nkd mutants that are heterozygous for wg, there is less ablation of denticle belts and the embryos appear more wild-type. Therefore the extent of naked cuticle produced in nkd mutant embryos depends critically on the gene dosage of wg. Even greater suppression of the nkd phenotype is obtained by reducing the dosages of en and hh, as well as wg (Fig. 4A,C).

en expression is also affected in nkd mutant embryos. The en expression domain is expanded 2 to 3 cells in a posterior direction during stages 8 and 9 (Fig. 6A,B; see also Martinez Arias et al., 1988). In the preceding section, we argue that the expanded en domain prevents activation of wg in the cells immediately posterior to the endogenous wg stripe. This exclusion of wg expression, which would reduce the total amount of naked cuticle specification activity in the segment, may account for the residual denticle belts observed in nkd single mutants (Fig. 4A). This view is consistent with the phenotype of the en nkd double mutant, which is more severe than the nkd single mutant phenotype (Fig. 1D). Only naked cuticle and no other pattern elements are visible on the ventral surface. This severe naked phenotype is not simply a failure to differentiate cuticular structures, as dorsal pattern elements differentiate normally (not shown). wg activity is entirely responsible for this enhanced naked phenotype. Removing wg activity, in the wg en; nkd triple mutant (Fig. 3D), restores a lawn of denticles to the ventral surface. The phenotype of the ptc en; nkd triple mutant (Fig. 3D) is similar to that of the en nkd double mutant, where only naked cuticle and no other pattern element is visible. Like en nkd, this pattern is due to wg activity, as denticles are restored to the cuticle pattern in wg ptc en; nkd mutants (Table 1).

patched and naked restrict wingless activity within the segment

Removal of ptc activity enhances the nkd mutant phenotype. The ptc; nkd double mutant produces only naked cuticle, with no ventral denticles visible (Fig. 1D). nkd mutants heterozygous for ptc also produce a completely naked cuticle (Fig. 4B). Since reducing ptc levels enhances the severity of the nkd phenotype, the wild-type ptc gene product probably functions to decrease the wg naked cuticle specification activity. The uniform naked cuticle produced by ptc; nkd double mutants suggests that all cells in the segment are exposed to wg. Eliminating wg activity, in the wg ptc; nkd triple mutant (Fig. 3C) restores denticles. As in nkd single mutants, the en expression domain is expanded in the ptc; nkd double mutant (Fig. 6C). Consequently wg expression is blocked in these cells, so that the wg expression domain in ptc; nkd mutants is similar to that of ptc single mutants (Fig. 5L). However, the boundaries of Wg protein distribution, which are very sharply defined in ptc and ptc en mutants (Fig. 5D), appear to be less sharply defined in ptc; nkd mutants (Table 1). This suggests that in the absence of nkd activity, Wg protein might be transported more effectively to parts of the segment outside of the wg expression domain. This enhanced distribution might also explain the apparently lower levels of Wg protein in the wg domain of ptc; nkd double mutants compared to ptc single mutants. In ptc and ptc en mutants, nkd gene activity restricts Wg protein movement and thus it accumulates to high levels in the wg transcription domain. Although our antibody preparations do not reveal detectable levels of Wg protein in cells outside of the wg domain of ptc; nkd double mutants or of nkd single mutants, we have shown that these cells are exposed to levels of wg activity sufficient to specify naked cuticle. We conclude that our antibody preparations cannot detect low levels of Wg protein that are functionally active by genetic criteria. Therefore, since the simplest means of restricting wg activity is by directly regulating Wg protein distribution, we propose that both ptc and nkd may act by altering movement or uptake of the Wg protein.

The expression pattern of nkd is not known, but our genetic data (above) show that it is required in the cells posterior to the wg stripe. In the absence of nkd gene product, these cells inappropriately express wg when en activity is not present to repress it. When en activity is present, the en expression domain expands in a posterior direction. This expansion depends on wg activity as it is delayed in nkd mutants that are heterozygous for wg (not shown) and it is absent in wg; nkd double mutants (Table 1). We propose that the en domain expands in response to inappropriate wg signalling, possibly due to increased Wg protein movement, and that wg activity extending to the line of cells posterior to the en-competent domain then induces wg expression in an autocatalytic fashion. This model demands only that wild-type nkd gene activity restricts wg activity; nkd need not act as a repressor of en expression as has been previously proposed (Martinez Arias et al., 1988; Dougan and DiNardo, 1992).

This model also explains a puzzling result obtained in
experiments where deregulated wg response is produced in all cells of the segment. Embryos that carry a heat-shock wingless construct, which provides ubiquitous wg expression, show expansion of the en expression domain (Nordermeer et al., 1992). A similar en expansion is observed in mutants for the zeste white 3 gene, which acts as a negative regulator of the wg response pathway (Siegfried et al., 1992). Both situations produce a phenotype identical to that of the ptc; nkd double mutant: expanded en expression and uniform naked cuticle specification. Our model would predict this expansion of the en domain as a consequence of uniform response to wg signal across the segment. The extent of cells competent to express en in response to wg signal may be defined by earlier pair-rule gene action, as proposed by Ingham et al. (1991). The width of the en domain in nkd and ptc; nkd mutants varies in a pair-rule fashion (Fig. 6B,C).

nkd also appears to modulate wg activity in cells anterior to the wg stripe. nkd mutants that are heterozygous for ptc show an anterior expansion of wg expression similar to the ptc; nkd double mutant (Fig. 7B,C), whereas ptc heterozygotes normally would show a wild-type pattern of wg expression (not shown). This unexpected ptc/+; nkd phenotype corresponds with the production of a cuticle indistinguishable from ptc; nkd, naked cuticle secreted by all cells of the segment (Fig. 4B). Therefore when nkd is absent, a reduction in the amount of ptc activity in the anterior cells may allow wg autoactivation in those cells. The ptc phenotype is subject to nkd dosage effects as well. ptc homozygotes that are heterozygous for nkd show a ptc-like pattern that is disrupted by greater expanses of naked cuticle (Fig. 4D,E). Thus, while both ptc and nkd are recessive mutations, each shows a dominant effect in homozygotes of the opposite mutation. This indicates that the dosage of each of these two gene products is critical for correct distribution of wg activity within the segment.

ptc encodes a transmembrane molecule that is localized to the cell surface (Nakano et al., 1989; Hidalgo and Ingham, 1990). Although nkd has not yet been molecularly characterized, it may also encode a cell surface molecule since it shows genetic properties similar to ptc. Both ptc and nkd can influence pattern in the absence of wg activity (described above), therefore we propose that these molecules alter some fundamental property of the cell membrane in which they are inserted. These cell surface properties may contribute to pattern in the absence of wg, but have their major effect on pattern via the wg signalling pathway. It is possible that these molecules restrict wg activity by regulating Wg protein transport and/or endocytosis. For instance, ptc and nkd may alter membrane properties such that endocytosis of the Wg protein is less efficient, thereby restricting the Wg protein distribution in wild-type embryos. Alternatively, they may be endocytosed as part of the membrane and provide tags that target the Wg-containing intracellular vesicles directly to the lysosome. In this way, Wg might be degraded before it can trigger a response in the cell, and it would not be transported effectively to cells further away from the endogenous wg-expressing stripe of cells.

During wild-type patterning, Wg protein distribution is dynamic and has different functional consequences (Bejsovec and Martinez Arias, 1991). Between 4 and 6 hours, Wg protein is present in cells on either side of the wg stripe; this corresponds with the time during which wg acts to specify the diverse denticle types in the segment and to

Fig. 7. Embryos doubly labelled with wg and en antibody staining. High magnification view of abdominal segments 5, 6, and 7 in the extended germ band (thus posterior is to the left). (A) In wild-type embryos, en (light gray nuclear staining) is expressed in a continuous 2-cell-wide stripe. wg (black punctate staining) is expressed in the row of cells immediately anterior to the en stripe, in a ventrolateral stripe and a dorsal spot. Arrowhead points to posterior end of ventral midline. (B) ptc; nkd (PN) double mutants show expansion of both the en and wg domains, although the dorsolateral repression of the wg stripe is still observed (bracket). All cells in the ventrolateral region are either expressing en or wg. (C) Embryos that are homozygous for nkd (N) and heterozygous for ptc (P/+) show not only the expanded en domain (bracket) characteristic of nkd single mutants, but also an expanded wg domain more typical of ptc homozygotes.
stabilize en expression in the cells posterior to the wg stripe. After 6 hours, Wg is distributed in a graded fashion in the cells anterior to the wg stripe; this corresponds with the time during which wg specifies naked cuticle identity. It is striking that Wg protein is detected at high levels in these anterior cells (Bejsovec and Martinez Arias, 1991) only after the time when their ptc expression levels decrease (Nakano et al., 1989; Hooper and Scott, 1989). Therefore we propose that the ptc and nkd molecules might play key roles in regulating the dynamic pattern of Wg protein distribution, which is crucial for the development of wild-type pattern.

Involvement of these molecules in the processing or interpretation of wg signal complicates the analysis of experiments examining ubiquitous wg transcription. Sampedro et al. (1993) conclude that wg does not act as a graded signal because denticle diversity is generated when heat shock-wg is expressed uniformly in a wg mutant embryo. However, our results indicate that the action of other segment polarity genes may contribute to the graded nature of wg signalling activity, and therefore uniformly transcribed wg may not actually produce uniform wg activity.

**ptc and hh show mutual suppression through opposing effects on wg expression**

The ptc: hh double mutant shows a partial suppression of the hh (Ingham et al., 1991) and also of the ptc single mutant phenotypes (Figs 1D, 3F). As with ptc single mutants, only two denticle types are produced in addition to naked cuticle. Row-1-type denticles occupy the anterior edge of the belts, as in wild-type and ptc, but the remainder of the belt is composed of fairly uniform row-3-type denticles, similar to those produced uniformly in wg ptc and wg ptc; hh mutants (Fig. 3A,G). At the posterior edge of the ptc; hh denticle belt, there is no mirror-image duplication of row 1. This causes the belt to appear more wild-type than those of the ptc single mutant. Unlike ptc single mutants (Fig. 6H), ptc; hh double mutants show no ectopic en stripe induced under the posterior edge of the belt (Fig. 6F). Thus no row-1-type denticles are specified inappropriately by these cells and no mirror-image duplication is observed. Unlike hh single mutants (Fig. 6G), ptc; hh double mutants show no decay of the endogenous en stripe: epidermal en expression in the ptc; hh mutant is indistinguishable from wild-type (Fig. 6E,F). The failure to form an ectopic en stripe may be due to the lower levels of wg expression observed in ptc; hh double mutants (Fig. 5E). Wg activity is required for production of the ectopic en stripe in ptc embryos, as it does not form in wg ptc double mutants (Table 1). The reduced amount of wg in ptc; hh double mutants may be insufficient to induce an ectopic en stripe.

It has been proposed that wild-type hh activity acts to maintain wg expression by antagonizing a transcriptional repressor of wg (Ingham et al., 1991). This postulated repressor molecule probably is not ptc (as proposed by Ingham et al., 1991), en or nkd, because hh affects wg expression in the absence of these three gene products. The wg domain in ptc; hh mutants, while expanded, is lower in intensity (described above) and does not appear as wide as it is in ptc single mutants (Fig. 5E,C). It is possible that the domain appears narrower because the endogenous stripe of wg still decays, as it would in hh single mutants (Fig. 5B).

A similar dependence of wg expression on hh activity is observed in ptc en; hh triple mutants: expanded wg domains form, as in ptc en double mutants, but they decay in alternate segments (Fig. 5N). This dependence is also observed in the ptc en; nkd hh quadruple mutant (Fig. 5O), where the uniform wg expression of ptc en; nkd (Fig. 5M) is now broken up into broad stripes, with some cells no longer expressing wg. In all three examples, hh affects wg expression in embryos that have no ptc activity.

**hedgehog alters the competence of cells to respond to wg signal**

The nkd hh double mutant also shows suppression of both the hh and the nkd single mutant phenotypes (Fig. 1D). nkd hh mutants are larger and produce more denticles than do nkd single mutants. nkd hh mutants are also larger and have a greater diversity of cell types and more pronounced segmental pattern than hh single mutants. This novel pattern consists of row 1 denticles followed by an expanse of naked cuticle that apparently replaces rows 2 through 4. The posterior portion of each segment is covered with row-5 and row-6-type denticles, mixed with intermediate type denticles. The presence of naked cuticle in nkd hh mutants is surprising because wg expression decays in these embryos during stages 8 and 9 (Fig. 5J), as it does in hh single mutants (Fig. 5B). In addition, en expression is expanded posteriorly (Fig. 6D), although it does not extend as far posteriorly as in nkd single mutants (Fig. 6B). Either undetectable levels of wg or the early transient wg expression in nkd hh embryos is responsible for both the specification of naked cuticle at the ventral midline and the stabilization of en expression. Both processes are eliminated in wg; nkd hh triple mutants (Fig. 3M; en decay, see Table 1). During normal development, early expression of wg would not be sufficient to direct these processes (see below; Bejsovec and Martinez Arias, 1991).

Similar effects on en maintenance are observed in the hh single mutant. In these embryos, en expression decays partially, but not completely (Fig. 6G). Some epidermal cells, particularly those at the ventral midline, retain stable en expression throughout development. This stable expression is due to wg activity, as it is eliminated in wg; hh double mutants (Fig. 6J). However, temperature-shift experiments removing wg activity at specific times during development indicate that wg activity is required between 5.5 and 6 hours to stabilize en expression in the ventral midline epidermal cells (Bejsovec and Martinez Arias, 1991). In hh mutants, wg expression decays early and is completely undetectable by 4.5 hours of development (Fig. 5B; see also Ingham and Hidalgo, 1993). Therefore either undetectable levels of wg mediate the en stabilization observed in hh and nkd hh mutants, or the absence of hh changes the timing of en stabilization by wg in these cells. Because we find that wild-type hh activity can block wg-specified denticle diversity in ptc en mutants (described above), we propose that hh might play a role in modulating the ability of cells to respond to wg signal at different times during development.

nkd hh, ptc; nkd hh and wg; nkd hh (Fig. 3K,L,M) show strong segmental sculpting of the lateral margin, suggesting an interaction with the dorsoventral patterning system. A
similar effect on the lateral margin is observed in hh single mutants that are heterozygous for ptc, or nkd, or both (Fig. 4F,G). Thus the balance among these molecules may affect the interplay between the anteroposterior and dorsoventral patterning systems. Much of the nkd hh mutant pattern depends on en activity. The en; nkd hh triple mutant pattern (Fig. 3N) shows less lateral sculpting and appears more defective than either the wg; nkd hh or ptc; nkd hh pattern. Row-5- and 6-type denticles are arranged continuously along the lateral margins of the denticle zone, with circular patches of naked cuticle that are continuous along the ventral midline. This naked cuticle is very sensitive to wg gene dosage. en; nkd hh mutants that are also heterozygous for wg (Fig. 3O) are large, but show virtually no segmental pattern. They produce only row-5-type denticles in a completely uniform lawn.

Maintenance of en expression does not always require wg activity

We have discovered an exception to the rule that wg is absolutely required to stabilize en expression. Unlike the wg ptc double mutant (Fig. 6K), the wg ptc; nkd triple mutant (Fig. 6L) shows stable en expression in some ventral and ventrolateral en cells, similar to that seen in hh mutants (Fig. 6G). hh gene activity is required for this wg ptc; nkd pattern of en expression. In wg ptc; nkd hh quadruple mutants, en expression decays during stages 8 and 9 (Table 1). Thus the consequence of being mutant for ptc; nkd renders en expression in some cells independent of wg activity but dependent on hh activity. This suggests that ptc, nkd and hh mediate some cell-cell interactions that critically depend on the balance among the three molecules. However, the consequences of en expression in wg ptc; nkd are irrelevant to the final cuticle pattern. Even though en expression is partially independent of wg function, no row-1-type denticles are specified. The wg ptc; nkd cuticle pattern is similar to the wg ptc en; nkd quadruple mutant phenotype (Table 1).

Patterning rules differ in the dorsal epidermis

Mutant effects on wg and en expression patterns differ between the dorsal and ventral surfaces. In hh mutants, en expression persists variably in ventral and ventrolateral portions of the stripe, and is lost elsewhere (Fig. 6G), while in nkd mutants, en expression is expanded uniformly in dorsal and ventral portions of the stripe (Fig. 6B). In nkd hh double mutants, expanded en expression is stably maintained only over the ventral and ventrolateral portions of the stripe; en expression decays in the more dorsal regions (Fig. 6D). Thus nkd is epistatic to hh only in the ventral and ventrolateral regions of the embryo. hh is epistatic to nkd in the dorsal and dorsolateral regions. In the ptc en; nkd hh quadruple mutant, the uniform expression of wg on the ventral surface seen in the ptc en; nkd triple mutant is broken up into broad stripes (Fig. 5O,M). However, the dorsal wg expression remains continuous across the segment. This indicates that hh plays a role in derepressing or promoting wg expression only on the ventral surface and not on the dorsal. Therefore in this case hh is epistatic to ptc en; nkd on the ventral surface and ptc en; nkd is epistatic to hh on the dorsal. We conclude that positional values in the dorsal epidermal cells are generated at least in part by rules different from those used in the ventral epidermal cells.

CONCLUSIONS

We have described the effects of removing five segment polarity gene activities, separately and in combination. This has allowed us to derive the following rules that govern patterning of the wild-type epidermis in Drosophila embryos:

1) The pattern is generated in part by a digital code where wg activity specifies naked cuticle cell identity and, in combination with en activity, specifies row-1-type denticles.

2) wg activity is required for generation of the diversity of cell types that comprise the remaining positional values within the segment. ptc, en, nkd and hh gene activities provide an inherent polarity that modulates the response of cells to this diversity-generating signal:

(a) Wild-type ptc and nkd gene activities are required to restrict wg activity within the segment, perhaps directly by regulating Wg protein distribution. The mutant phenotypes of both ptc and nkd can be explained by unrestricted wg activity producing an autocatalytic effect on wg expression in inappropriate regions of the segment.

(b) Wild-type hh activity may act to restrict or delay the competence of cells to respond to the wg signal. In ptc en mutants, wild-type hh activity appears to inhibit the generation of diverse cell types by wg signalling. In hh and nkd hh mutants, normal responses to wg signalling - specification of naked cuticle and/or maintenance of en expression - may occur at inappropriate early times.

3) ptc, en, nkd and hh gene activities are required for the pathway by which wg generates diverse positional values within the segment, but they are not required for the pathway by which wg activity causes cells to secrete naked cuticle. In ptc; nkd, en; nkd and ptc en; nkd mutants, naked cuticle is specified across the entire ventral epidermis. The embryos are also small and highly defective. Removing wg activity in the triple mutants wg ptc; nkd, wg en; nkd and the quadruple mutant wg ptc en; nkd results in embryos that are similar in size and defect, but that now have denticles. The size and general appearance of the mutant embryos suggest that wg activity does not alter the total amount of positional information, but rather alters the decision of cells to secrete naked cuticle as opposed to uniform denticles. Thus the pathway by which wg acts to generate diversity may be distinct from the pathway by which it specifies naked cuticle.

4) ptc, en, nkd and hh gene activities also can contribute to pattern through a pathway independent of the wg signalling pathway, as each alters epidermal pattern in the absence of wg activity.

5) en cells produce (at least) 2 signals:

(a) One signal is encoded by hh. This signal acts on adjacent cells to maintain wg expression early in development, possibly by antagonizing a repressor molecule in cells of the wg-expressing stripe. This repressor cannot be ptc exclusively because hh gene activity affects the expression of wg even in the absence of ptc.
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