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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 (wg^{CX4} and en^{SFX31}) or are the strongest alleles available (ptc^{IN108}, nkd^{7E89} and hh^{GS1}). Other alleles used for comparison were wg^{IG22}, ptc^{6P43}, ptc^{IF85}, en^{IK57}, en^{IM99}, hh^{6N16} and hh^{13C29}. 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. 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 wg^{CX4} *(W)*, ptc^{IN108} *(P)*, en^{SFX31}*(E)*, nkd^{7E89} *(N)* and hh^{GS1}*(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-

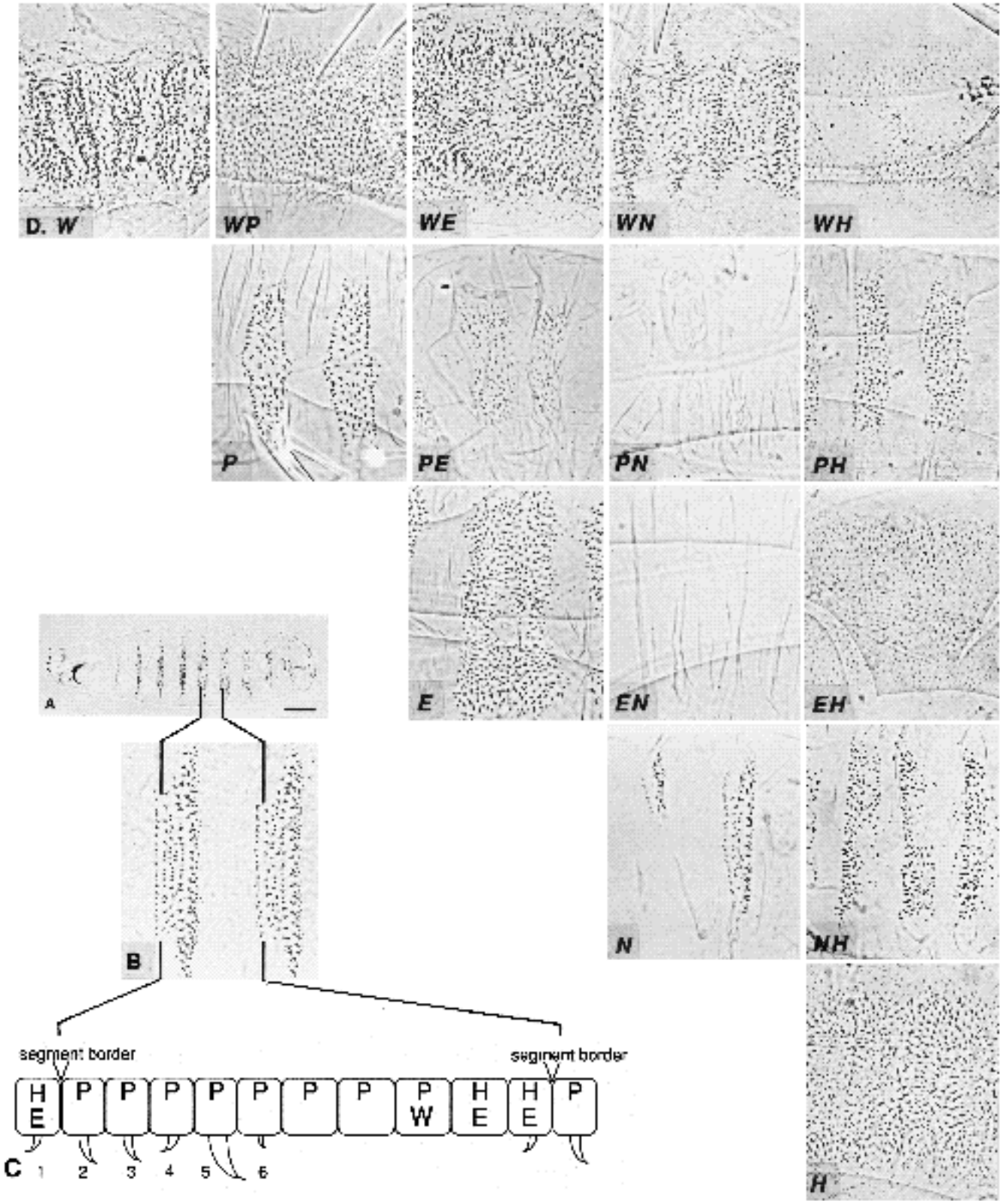

Fig. 1

Table 1. Summary of single and multiple mutant phenotypes

Genotype	Denticle type	Ventral denticle pattern	*wg* distribution	*en* distribution
Wild-type	N, 1 - 6	23456NNNN1	OOOOOOOΘOO	OOOOOOOOΘΘ
W	5	lawn with segmental polarity reversals	decays at stage 10	decays at stage 9
P	N, 1, 2	22221NNNN1	OOOOΘΘΘΘOO (high levels,sharp edges)	OOOΘOOOOΘΘ ectopic at stage 10
E	N, 2 - 6	lawn with naked patches in alternate segments	decays at stage 10 (pair-rule modulation)	n.d.
N	N, 1 - 6	belts missing in alternate segments	OOOΘOOOΘOO ectopic at stage 10	ΘΘΘOOOOOΘΘ
H	5	disorganized lawn with central whorls	decays at early stage 9 (pair-rule modulation)	persists in ventral and dorsolateral spots
WP	3	segmental lateral whorls	like *W*	like *W* (CNS like *P*)
WE	5	lawn with central whorls	like *W*	n.d.
*WN**	5, i	weak *W* (37/252, χ^2=.246, 2 d.f.)	like *W* (72/283, χ^2=.074, 2 d.f.)	like *W* (87/385, χ^2=.345, 2 d.f.)
*WH**	5	severe *H* (29/272, χ^2=.533, 2 d.f.)	n.d.	like *W* (CNS like *H*) (39/595, χ^2=.062, 3 d.f.)
PE	2	22222NNNN	like *P*	n.d.
*PN**	N	only naked cuticle (77/194, χ^2=.307, 2 d.f.)	like *P* but edges not sharp (32/464, χ^2=1.48, 3 d.f.)	ΘΘΘΘOOOOΘΘ (21/615, χ^2=.697, 3 d.f.)
*PH**	N, 1, 3, 4	333334NNN1 (12/98, χ^2=.143, 2 d.f.)	like *P* but levels not high (30/550, χ^2=.082, 3 d.f.)	like wild-type (CNS like *P*) (43/608, χ^2=.056, 3 d.f.)
*EN**	N	only naked cuticle (87/526, χ^2=.624, 2 d.f.)	ΘΘΘOOOOOOΘ some decay at stage 11 (pair-rule modulation) (39/498, χ^2=.468, 2 d.f.)	n.d.
*EH**	5	severe *H* (44/355, χ^2=.952, 2 d.f.)	n.d.	n.d.
NH	N, 1, 5, 6, i	NNN55555/61	decays at stage 10	like *N*
WPE	2	unpatterned lawn	n.d.	n.d.
*WPN**	2	disorganized *WP* (48/625, χ^2=.554, 5 d.f.)	n.d.	like *H* (CNS like *WP*) (16/450, χ^2=.200, 4 d.f.)
*WPH**	3	like *WP*, but embryo larger (32/429, χ^2=.771, 5 d.f.)	n.d.	like *W* (CNS as well) (132/575, χ^2=.523, 3 d.f.)
*WEN**	5	like *WE* (170/484, χ^2=1.80, 4 d.f.)	n.d.	n.d.
*WEH**	5	severe *H* (101/940, χ^2= 1.55, 4 d.f.)	n.d.	n.d.
*PEN**	N	only naked cuticle (107/668, χ^2=.775, 4 d.f.)	like *P* until stage 10, then ΘΘΘΘΘΘΘΘΘΘ (35/631, χ^2=.146, 4 d.f.)	n.d.
*PEH**	N, 2 - 6	2333(45)3NNN some belts fused in pairs (112/921, χ^2=.364, 5 d.f.)	like *P* until stage 11, then alternate stripes decay (29/441, χ^2=.113, 4 d.f.)	n.d.
*WNH**	5, i	like *NH* with no naked (116/1277, χ^2=.673, 4 d.f.)	n.d.	like *WH* (CNS like *H*) (41/595, χ^2=.171, 4 d.f.)
*PNH**	N, 1, 5, 6, i	like *NH* (151/488, χ^2=1.05, 4 d.f.)	like *NH* (106/480, χ^2=.271, 4 d.f.)	like *NH* (16/99, χ^2=.250, 5 d.f.)
*ENH**	N, 5, 6	naked at midline, belt edges fused laterally (31/295, χ^2=.525, 5 d.f.)	like *NH* (131/437, χ^2=.153, 4 d.f.)	n.d.
*WPEN**	2	like *WPE* (253/673, χ^2=.409, 5 d.f.)	n.d.	n.d.
*WPEH**	2	like *WPE* (465/1310, χ^2=.246, 4 d.f.)	n.d.	n.d.
*WPNH**	3	like *WP* (414/1166, χ^2=.639, 6 d.f.)	n.d.	like *WP* (60/195, χ^2=.529, 7 d.f.)
*WENH**	5	like *WE* (365/1076, χ^2=.466, 6 d.f.)	n.d.	n.d.
*PENH**	N	only naked cuticle (88/765, χ^2=.603, 5 d.f.)	broad segmental domains ventrally, uniform dorsally (14/320, χ^2=.865, 6 d.f.)	n.d.

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. 'Denticle 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^{IL114}, 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%.

tinguishable 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.

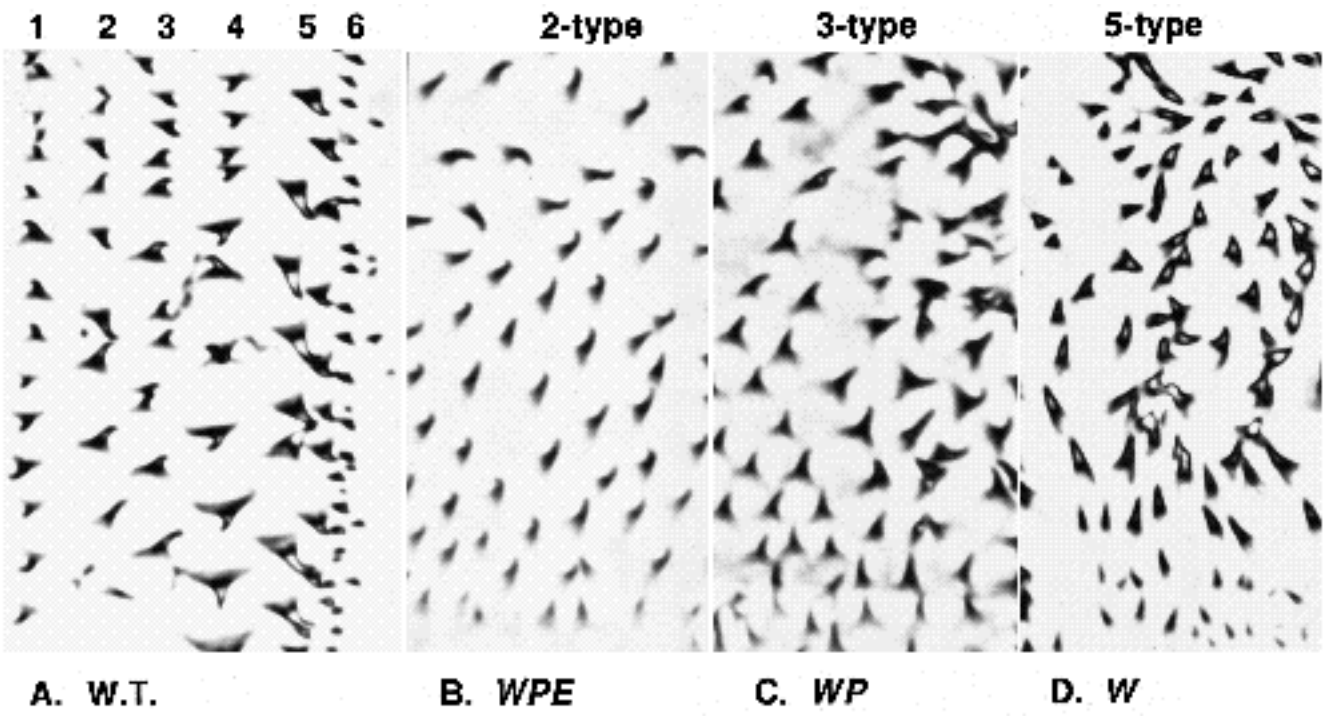

Fig. 2. Assignment of denticle type identities. (A) High-magnification view of half of a wild-type denticle belt, showing the denticle types characteristic of rows 1 through 6. Ventral midline is at top, ventrolateral edge is at bottom. Because denticle morphologies in rows 2 through 4 vary from ventral 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.

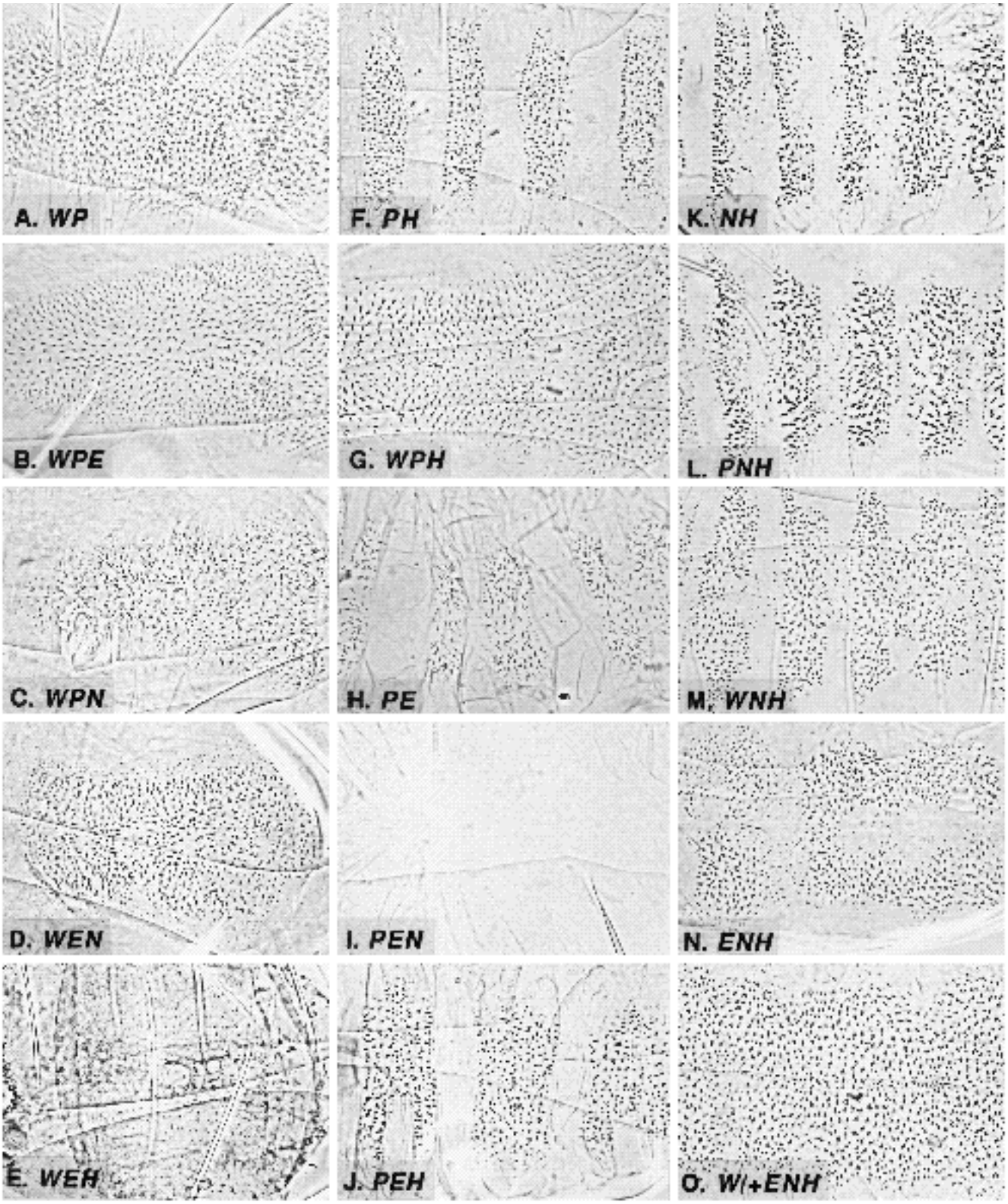

Fig. 3. Cuticle patterns of doubly and triply mutant embryos. Genotypes are designated as: $W = wg^{CX4}$, $E = en^{SFX31}$, $P = ptc^{IN108}$, $N = nkd^{7E89}$ and $H = hh^{GS1}$. See Table 1 for assessment of denticle type identities and statistical significance of unbalanced phenotypes.

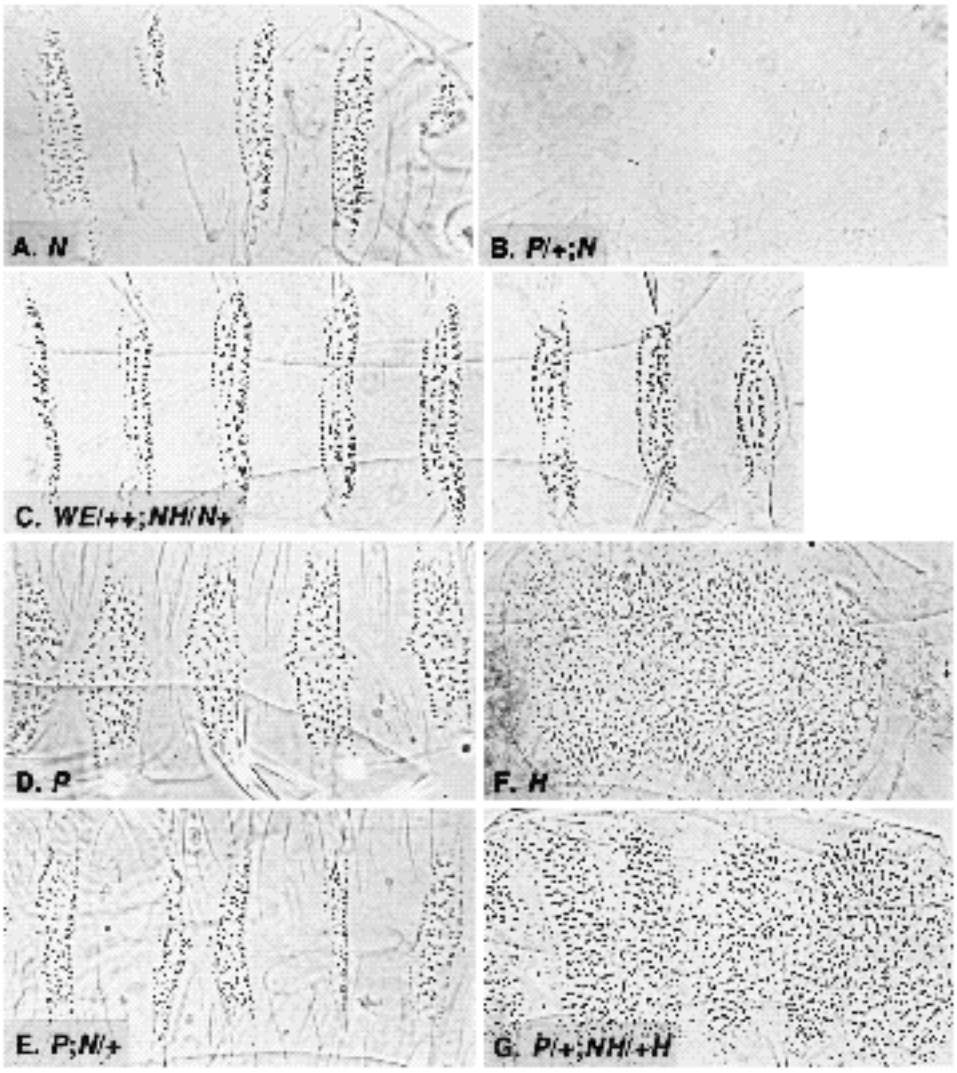

Fig. 4. Cuticle patterns of single mutants that are heterozygous for other mutations. (A) *nkd(N)* homozygote in a wild-type background. The entire ventral pattern of this mutant embryo is shown. nkd^{7E89} homozygotes are much smaller than wild-type and typically secrete three complete denticle belts and a variable number of partial belts. (B) *nkd (N)* homozygote heterozygous for *ptc (P*/+). This phenotype is indistinguishable from that of the *ptc; nkd* double mutant. The embryos are smaller than the *nkd* single mutant alone. (C) *nkd (N)* homozygote heterozygous for *wg en (WE*/++), and *hh (H*/+). These embryos are virtually wild type in size and show eight complete denticle belts, but the embryos still suffer the severe head defects typical of *nkd* homozygotes. (D) *ptc (P)* homozygote in a wild-type background. (E) *ptc (P)* homozygote heterozygous for *nkd (N*/+). These embryos are comparable in size to the *ptc* single mutant. (F) *hh (H)* homozygote in a wild-type background. (G) *hh (H)* homozygote heterozygous for *ptc (P*/+) and *nkd (N*/+). These embryos are larger than the *hh* single mutant alone and their denticle pattern slightly resembles that of the *nkd hh* double mutant (Fig. 3K) due to segmental sculpting of the lateral margin.

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.

engrailed specifies row-1-type denticles

The *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 *engrailed* fail to produce row-1-type denticles, demonstrating that *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 *en* single mutants can account for the pattern of naked cuticle produced in alternate segments. *wg*-expressing cells require a signal from the adjacent *en/hh*-expressing cells in order to maintain their *wg* expression (Martinez Arias et al., 1988). In *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 *en* mutants. The naked cuticle patches are clearly due to *wg* activity as they are absent in *wg en* double mutants (Fig. 1D), but *wg* does more than just specify naked cuticle in *en* mutants. In *wg en* double mutants, only row-5-type denticles are produced, as opposed to the many denticle types present in *en* single mutants. Thus *wg* must contribute to the specification of denticle types 2, 3, 4 and 6.

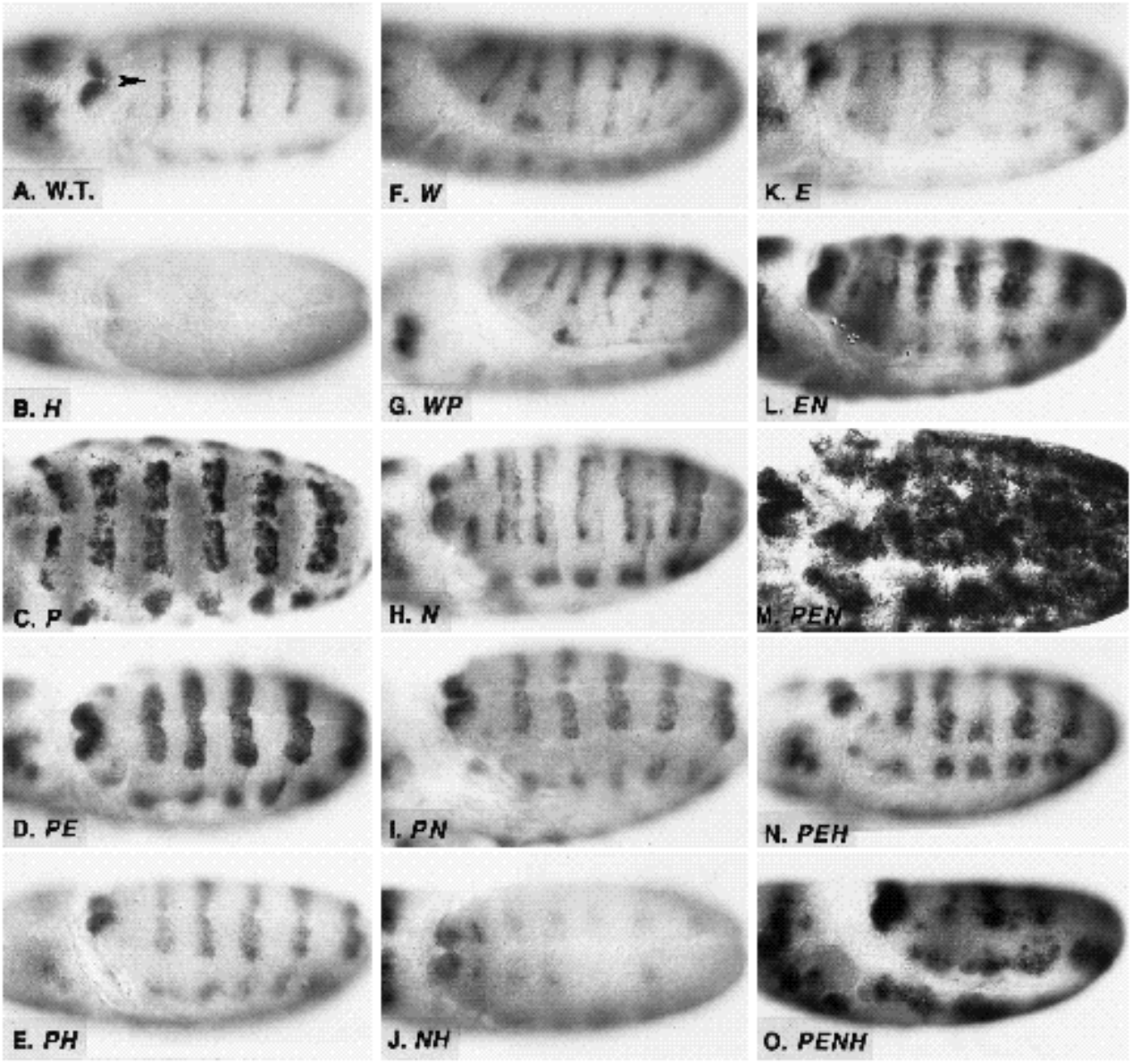

Fig. 5. *wg* expression in wild-type and mutant embryos. Wg protein distribution or *wg* RNA (C and M) are shown in abdominal segments 4 through 8 of extended germ band embryos. Embryos are oriented with anterior to the left and ventral side down, but due to curvature of the germ band, the ventral surface 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^{IL114}, makes a protein that is stable but not functional. (G) *wg ptc* double mutants carrying the wg^{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.

engrailed and *hedgehog* contribute to pattern independently

The *hh* single mutant phenotype consists of row-5-type denticles arranged in a disorganized pattern of ventral midline whorls, with no naked cuticle (Fig. 1D, 4F; see also Nüsslein-Volhard and Wieschaus, 1980; Mohler, 1988). This phenotype resembles that of the *wg en* double mutant (Fig. 1D). *hh* and *en* are expressed in the same set of cells, even in mutants where *en* expression is induced ectopically (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 hh* 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. 3J) 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^{IL114} 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 autocatalytic 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 autocatalytic 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

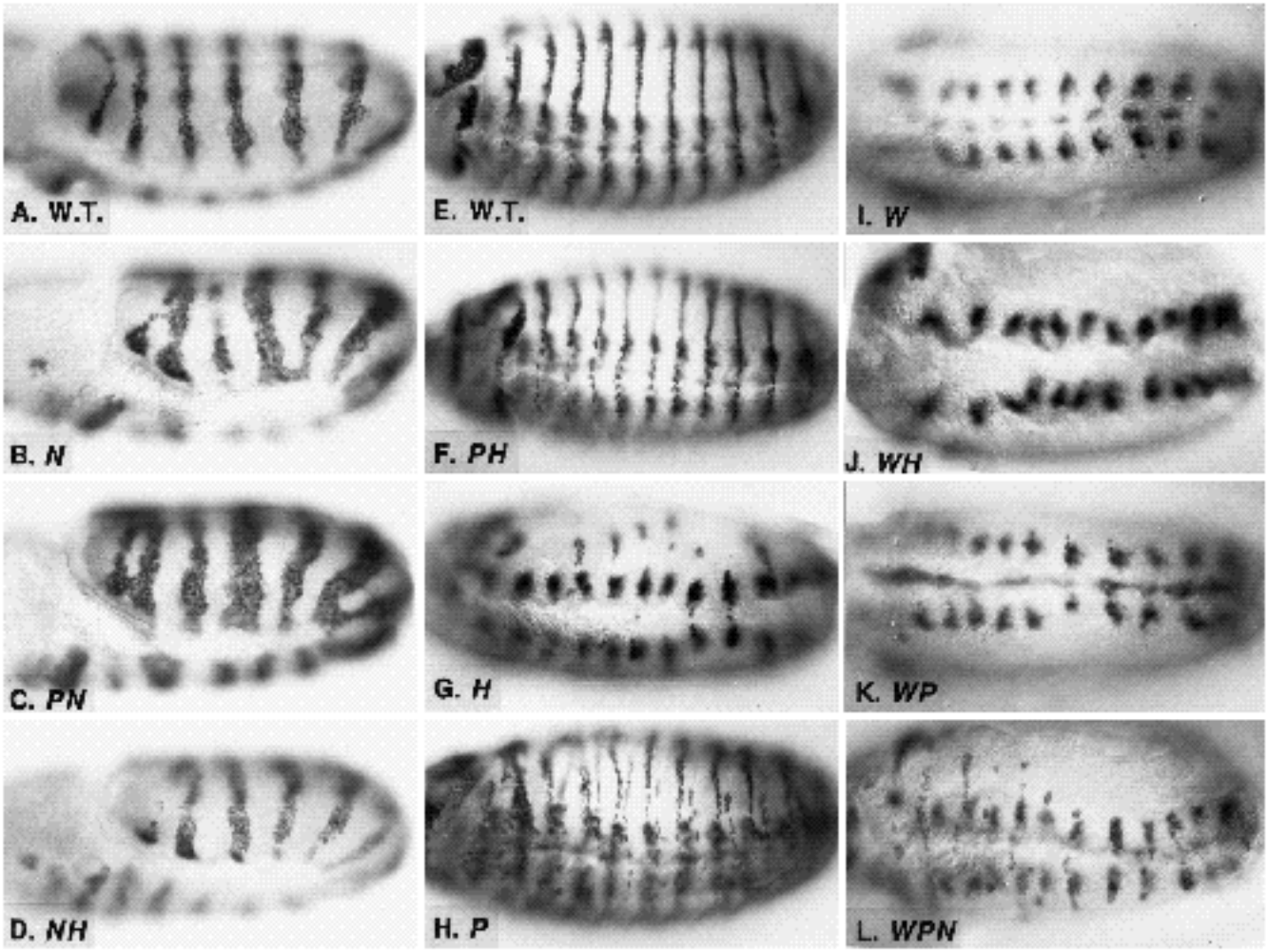

Fig. 6. *en* expression in wild-type and mutant embryos. (A) At stage 11, stripes of 2 to 3 *en*-expressing cells are seen in each wild-type segment. (B) In *nkd* mutants, the *en*-expressing domain is expanded in a posterior direction: even-numbered *en* stripes are 4 to 5 cells wide, odd-numbered *en* 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 en; 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. 3I) 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. 5I,C). 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

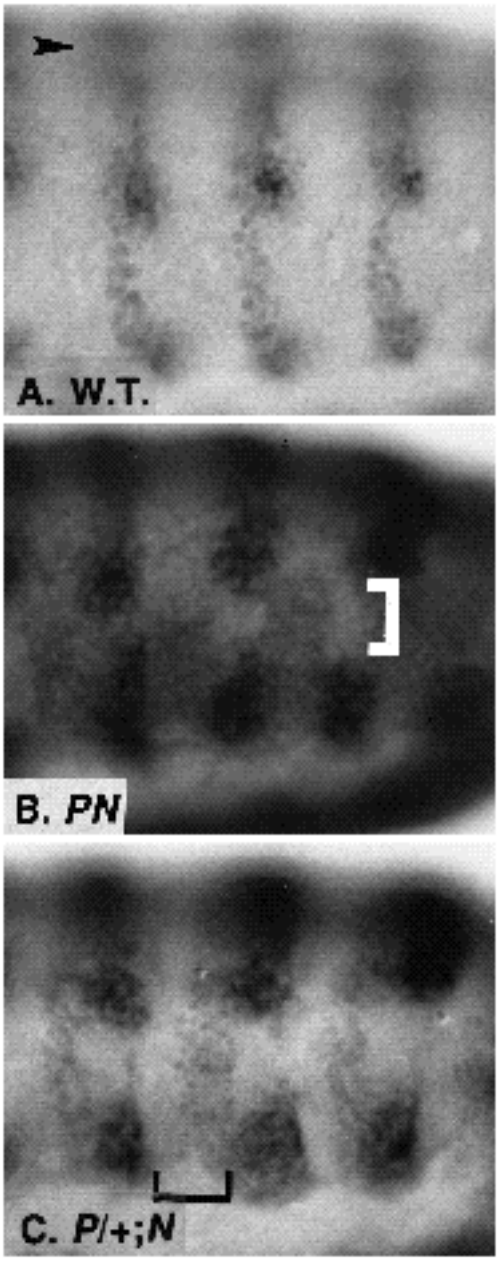

Fig. 7. Embryos doubly labelled with *wg* and *en* antibody staining. High magnification view of abdominal segments 5, 6, and 7 in the extended germ band (thus posterior is to the left). (A) In wild-type embryos, *en* (light gray nuclear staining) is expressed in a continuous 2-cell-wide stripe. *wg* (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* double 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. 6I,J). 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 continous 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 denticle cell identity.
(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 inappropriately 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*.

(b) The other signal is generated by *en* gene activity with no input from *hh*. This *en* signal is required to generate the correct orientation of denticles perpendicular to the antero-posterior axis and can act to specify row 3 cell type identity across the entire segment in the absence of *wg* and *ptc* activity.
(6) Positional values within the denticle belt need not be specified in a sequential order. In *ptc; hh* mutants, row-1- and row-3-type denticles can be specified without the intervening row-2-type denticles. In *nkd hh* mutants, row-5- and 6-type identities can be specified without rows 2 through 4. Thus patterning is not progressive. Each row of cells appears to measure the balance among the patterning molecules and make an absolute decision about positional identity.
(7) Patterning rules differ on the dorsal and ventral surfaces. In addition, the lateral edges of the ventral denticle expanse show dramatic modulation in different segment polarity mutant combinations, indicating an interplay of anteroposterior patterning with the dorsoventral patterning system.

We conclude that *ptc*, *en*, *nkd* and *hh* are important in restricting *wg* signalling and/or modulating response to *wg* signalling within the segment. This is consistent with the idea that a gradient of *wg* activity specifies positional values across the segment, with diverse cell fates being assigned in response to different threshold levels of *wg*. However, our results also indicate that *wg* level alone does not specify individual cell identities. Specific cell types can be produced in the absence of *wg*, for instance row 3 cell identities are observed in *wg ptc* mutants and row 2 cell identities in *wg ptc en* mutants. If *wg* level alone conferred these positional values, all mutant combinations lacking *wg* should show the same row-5-type identity as the *wg* null mutant. Since similar cell fates are arrived at by apparently different mechanisms and are not necessarily specified in sequential order, it is possible that *ptc*, *en*, *nkd* and *hh* have independent inputs into the final assignment of cell identity. Any hypothesis for how these molecules specify pattern must consider the observation that each can influence pattern in the absence of *wg*, in addition to their dramatic effect on the *wg* signalling pathway. For instance, *ptc*, *en*, *nkd* and *hh* might contribute to pattern formation by altering some fundamental cell surface properties, such as adhesivity, which then strongly influence the *wg* signalling pathway.

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