The roles of hedgehog, wingless and lines in patterning the dorsal epidermis in Drosophila

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SUMMARY

Rows of cells that flank the parasegment boundary make up a signaling center within the epidermis of the Drosophila embryo. Signals emanating from these cells, encoded by hedgehog (hh) and wingless (wg), are shown to be required for all segment pattern dorsally. Wg activity is required for the differentiation of one cell type, constituting half the parasegment. The gene lines appears to act in parallel to the Wg pathway in the elaboration of this cell type. Hh activity is responsible for three other cell types in the parasegment. Some cell types are specified as Hh activity and interfere with the function of patched, analogous to patterning of imaginal discs. However, some pattern is independent of the antagonism of patched by Hh, and relies instead on novel interactions with lines. Lastly, we provide evidence that decapentaplegic does not mediate patterning by Hh in the dorsal epidermis.

Key words: Hedgehog, Wingless, Lines, segment polarity, Drosophila

INTRODUCTION

During development, pattern must be established across fields of cells. This involves the instruction of cell fates by signals emitted from neighboring cells. The Drosophila wg and hh genes encode two such signals. For instance, both Hh and Wg signaling help organize leg bristle pattern (Baker, 1988; Couso et al., 1993; Struhl and Basler, 1993; Basler and Struhl, 1994; Couso et al., 1994; Tabata and Kornberg, 1994; Wilder and Perrimon, 1995). wg encodes a secreted glycoprotein of the conserved Wnt family (Cabrera et al., 1987; Rijswijik et al., 1987; Nusse and Varmus, 1992), and hh encodes a novel secreted protein that undergoes autoproteolytic activation (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Lee et al., 1994; Porter et al., 1995). In amphibians, a Wnt-related molecule may play an early role in axial patterning as a Nieuwkoop signal, while murine Wnt-1 is essential for mid-hindbrain development (McMahon and Moon, 1989; Chakrabarti et al., 1992; McMahon et al., 1992). Vertebrate Shh has been strongly implicated as a signal in limb, somite and neural tube patterning (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Fan and Tessier-Lavigne, 1994; Roelink et al., 1994). The widespread use of Wg, Hh and their relatives during pattern formation has sparked interest in their mechanisms of action, some of which are being revealed in the studies of Drosophila embryonic segment pattern.

wg and hh are expressed in adjacent cells that flank each parasegment boundary (Baker, 1987; Lawrence et al., 1987; Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992). Cells expressing hh also express engrailed (en), which helps maintain hh expression (Tabata et al., 1992). Although wg and hh are expressed in narrow domains, null mutations in either gene cause the global loss of segment pattern (Nusslein-Volhard and Wieschaus, 1980; Baker, 1988; Mohler, 1988). Null mutations in either gene also compromise the function of the other because reciprocal signaling between the wg and hh expressing cells is required to maintain each other’s expression (DiNardo et al., 1988; Martinez Arias et al., 1988; Tabata et al., 1992; Cumberledge and Krasnow, 1993; Ingham and Hidalgo, 1993). Thus, it is difficult to draw specific conclusions concerning the role of Wg or Hh in specification of cell fate from consideration of the null mutants alone. However, reciprocal signaling is only required for a finite time. Hh-expressing cells only require Wg input between 3 and 6 hours of embryonic development (Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991). The maintenance of wg expression in the dorsal epidermis only requires Hh input during the same interval (Heemskerk and DiNardo, 1994). Therefore, one can use temperature-sensitive alleles to test the distinct contributions of Wg and Hh by supplying early function, but removing later function, and observing effects on patterning. Such studies have led to proposed roles for Wg in patterning the ventral epidermis and for Hh in patterning the dorsal epidermis (Baker, 1988; Bejsovec and Martinez-Arias, 1991; Heemskerk and DiNardo, 1994). Here we explore the role of Wg in the dorsal epidermis, while extending analysis of Hh.

We previously showed that different concentrations of Hh specify distinct cell types within the dorsal epidermis (Heemskerk and DiNardo, 1994). Recent experiments attempted to increase the level of Hh from the source using the
binocular GAL4-UAS system, and resulted in little or no affect on long-range pattern (Fietz et al., 1995). Thus, although Hh can act as a morphogen, it appears not to do so normally. Perhaps the graded requirement for Hh activity that we have demonstrated in the dorsal epidermis is mediated by other genes through which Hh is thought to act, such as ptc and dpp.

Dpp mediates the role of Hh in patterning the eye and wing, and also a portion of the leg. In these cases, Hh-expressing cells signal locally to neighbor cells, activating the expression of dpp (Campbell et al., 1993; Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). If hh is ectopically expressed, growth and pattern occur ectopically, concomitant with the induction of dpp in neighboring cells (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Fietz et al., 1995). Importantly, ectopic expression of dpp is sufficient to cause this reorganization of growth and pattern independently of Hh (Diaz-Benjumea et al., 1994; Zecca et al., 1995).

Another consequence of hh signaling is the antagonism of ptc gene activity. Defects in patterning the ventral embryonic epidermis caused by null mutations in the hh gene are largely reversed by the combined inactivation of ptc and hh (Ingham et al., 1991). This antagonism is also seen in leg, wing and eye patterning (Capdevila et al., 1994; Hidalgo, 1994; Felsenfeld and Kennison, 1995; Heberlein et al., 1995; Lepage et al., 1995).

Here we demonstrate that although Hh acts in a graded manner to specify distinct cell types, the mechanism by which it acts is not through Dpp, nor entirely through antagonism of Ptc. Instead, part of the pattern arises through novel interactions between Hh and the gene lines. In addition, Wg signaling is required for the remaining cell types in the dorsal epidermis, and acts in parallel with lines to execute this role.

MATERIALS AND METHODS

Fly stocks

Strains are listed in Lindsley and Zimm (1992). wgts embryos are from wgts / Sm6B stocks; hhts embryos are from the cross: hhts Ubxts / MKRS x hh13C Ubxts / MKRS. ptc mutant embryos were heterallelic ptc5N / Df(2R)44C-E. The ptc; hh double mutants were derived from the cross: ptc5N / CyO; hh13C Ubxts / MKRS x Df(2R)44C-E / CyO; hh13C Ubxts / MKRS. Both lin2ts and lin2ts have strong and possibly null phenotypes, as homozygotes are phenotypically similar to each allele over deficiency Df (2R)G75 44F (4,5) - 44F (10,11).

Mutant analysis

The ptc phenotype is somewhat variable. For instance, dorsally there is often a duplication of 1° cell types usually appearing as a swirl of large denticles approaching and then veering away from the dorsal midline (Hooper and Scott, 1989), Fig. 4B). This duplication is missing in parasegments of some ptc mutant embryos. The ectopic induction of en (and hh) expression is similarly variable (DiNardo et al., 1988; Martinez Arias et al., 1988). In our experiments, ptc; hh double mutants are identified by Ubx. The homeotic transformation sometimes requires careful inspection in a ptc mutant background due to variability in the position of the mirror image within the segment. The distribution of WT / ptc : hh ; ptc ; hh was as expected (127 : 29 : 34 : 15). In the ptc ; hh embryos, the swirl of duplicated 1° cell types was most often suppressed, although in one parasegment in each of two double mutants there were still ectopic 1° denticles. In the double mutants there were usually no 3° cell types, except in three cases,
where there were groups of 3, 3 and 1 denticles, respectively, that had the appearance of a 3° cell type.

**Temperature shift and heat shock**

Cellularizing embryos were picked onto agar plates, aged at the appropriate temperature, and either shifted from 16°C to 29°C, or heat-shocked at 37°C for the appropriate interval. Embryos were aged either for 1.5 hours before fixation and staining, or until hatching for cuticle preparations. Developmental times listed are the mid-point of the approx. 45 minutes age spread, and have been adjusted for slower growth at lower temperatures.

Note that contrary to previous reports, the activity of the temperature-sensitive wg allele is not fully wild type. Raising embryos at 16°C still generated reproducible phenotypes dorsally (Fig. 11), and mild but also reproducible phenotypes ventrally (extra denticles near the midline). Heteroallelic combinations of wg with null alleles exhibited much worse defects, even when grown at 16°C.

Both HS-GAL4 and Ptc-GAL4 have been used previously to drive Hh expression, and alter fates, within the embryonic dorsal and ventral epidermis (Fietz et al., 1995). In our experiments Ptc-GAL4 / UAS-Dpp leads to strong expression ventrally and, during germband retraction, to stripes flanking the en stripes in the dorsal epidermis. Except for some gaps in the dpp stripes dorsally, this is the expected pattern of ptc transcription (Hooper and Scott, 1989; Nakano et al., 1989). The level of ectopic expression dorsally approximated that of the normal dpp-expressing cells at the edge of the epidermis. The earlier ventral dpp expression caused all ventral and ventrolateral fates to be replaced by dorsolateral and dorsal fates, as expected (Staehling-Hampton et al., 1994). Embryos carrying both HS-GAL4 and UAS-dpp, and that were heat-shocked for 25 or 40 minutes at 6, 7 or 8 hours after egg laying (AEL), also had shifts in the dorsoventral fate map, although not as extensive as with Ptc-GAL4, since we induced ectopic dpp expression at later times.

**Embryo manipulations**

Cuticle preparations were not baked (van der Meer, 1977; Wieschaus and Nüsslein-Volhard, 1986). RNA probes were synthesized from dpp, hh or wg templates (Baker, 1987; Padgett et al., 1987; Tautz and Peifer, 1989; Lee et al., 1992). Double labeling was as described by Dougan and DiNardo (1992). Monoclonal 4D9 was used to visualize en / inv (Patel et al., 1989); anti-β-galactosidase was from Cappel. Standard lacZ-activity stains were performed (Heemskerk and DiNardo, 1994).

**RESULTS**

**Wg and Hh signaling account for all cell types across the dorsal epidermis**

There are four different cell types along a segment dorsally (Fig. 1A). A single row of cells produces large pigmented denticles (1°). This is followed by about two rows of cells that secrete smooth cuticle (2°). The next two or three rows of cells produce thick pigmented hairs (3°) that are smaller and less pigmented than the 1° cell type. Posterior to this, several rows of cells produce about seven rows of fine hairs (4°). Another region of smooth cuticle lies posterior to 4° cells. Two rows of

![Fig. 2. dpp expression in WT. Dorsal to top, anterior left for all embryos.](image)

- **A:** WT 4.5 hours AEL, expression of dpp RNA (blue). (B) WT 6.5 hours AEL, doubly labeled to visualize dpp RNA (blue) and En (brown nuclei), which is expressed in stripes that mark the cells expressing hh. Bars are 50 μm.

![Fig. 3. Ectopic hh expression can induce ectopic dpp expression, but dpp is not sufficient to reorganize pattern.](image)

- (A) WT, as in Fig. 1A. (B) HS-Hh+/+, 5 minute heat shock at 8 hours AEL. With hh supplied to all cells, there is an increase in 3° cell types, from two to about four rows, at the expense of 4° cell types, from seven to about four rows. (C) HS-hh/HS-hh, 20 minute heatshock at 8 hours AEL. Higher level of hh causes the 3° and some 4° cells to adopt 2° fates. Occasional 3° cell types remain among those that adopt 2° fate. (D) UAS-dpp ; HS-GAL4, heat-shocked at 6 hours AEL. (E-G) 9.5 hours AEL, doubly labeled to visualize En protein (brown) and dpp RNA (blue). (E) HS-hh/HS-hh, no heat shock. Note normal dpp expression at edge of dorsal epidermis (arrow). (F) Magnified view of E. (G) HS-hh/HS-hh, 40 minute heat shock, 8 hours AEL. dpp expression is induced in a stripe located two cell diameters posterior to the en/hh-expressing cells. (H) UAS-dpp ; HS-GAL4, 40 minute heat-shock 6 hours AEL, fixed and stained 1.5 hours later. dpp is ectopically expressed (arrowhead) in patches throughout embryo (dorsal epidermis indicated between arrows). Compare to lack of dpp expression at same stage in WT (Fig. 2B). Bars are 10 μm in A-D, 50 μm in E-H.
cells express en/hh, the posterioriormost of which are the 1° cell types (Heemskerk and DiNardo, 1994). wg is expressed in cells anteriorly adjacent to the en/hh-expressing cells, in cells that adopt the 4° fate (Fig. 1E).

Inactivation of Hh function at 6 hours AEL using a hhTS allele leads to the loss of 1°, 2° and 3° cell types, but not 4° cell types (Heemskerk and DiNardo, 1994; Fig. 1D). The requirement is graded, since inactivation at 7 hours AEL leads to loss of 1° and 2° cell types (Fig. 1C), while inactivating at 8 hours AEL leads to the loss of only 1° cell types (Fig. 1B). There is no apparent requirement for Hh in the specification of 4° cell types. Embryos null for hh function still have 4° cell types (data not shown). Since wg is expressed in some cells that adopt 4° fates, we asked if Wg activity was required for these fates.

Wild-type embryos have 7 rows of fine hairs (Fig. 1A; WT). When wgts embryos are raised at the permissive temperature the segment has an average of 5.5 rows of fine hairs (Fig. 1I, 16°C). Thus, the wgts allele provides close to wild-type levels of Wg activity (see Materials and Methods). If Wg function is inactivated at 6 hours AEL nearly all 4° cell types are abolished (Fig. 1H, I). Progressively later inactivations allow 2.5 (7 hours; Fig. 1G, I), 3-4 (8 hours; Fig. 1F), and more than 4 (9 hours; Fig. 1I) rows of 4° cell types to be specified. Shifts after 9 hours are identical to those raised only at the permissive temperature. Also, progressively earlier loss of Wg function leads to progressively smaller segment size compared to wild type, suggesting that the cells that would normally adopt the 4° fate die in the absence of Wg function. Thus, Wg signaling is required for the 4° cell types since the longer Wg is active, the more rows of cells survive and differentiate into 4° cells. Furthermore, in each of the shifts, the 1°, 2° and 3° cell fates are established normally. We conclude that Hh and Wg activity are required in two adjacent domains of cells, which together constitute the entire array of dorsal cell types. These signaling pathways operate between 6 and 9 hours AEL (Fig. 7A). We next explore further the role of Hh in patternning the dorsal epidermis.

Fig. 4. 1° and 2° cell types are specified by the antagonistic relationship between hh and ptc. (A) Wild type (WT). (B) ptcIN / Df(2R)44CE. 3° cell types are missing, as are probably some 2° cell types normally positioned anterior to the 3° cells. 2° and 3° cells are replaced by a duplication of large pigmented denticles that are likely to be 1° cell types, with smooth cuticle between the duplicated elements. In ptc mutants, wg gene expression expands anteriorly, and there is an ectopic induction of en/hh expressing cells just anterior to this (DiNardo et al., 1988; Martinez Arias et al., 1988). These changes in gene expression correlate with the presence of the duplicated 1° cell types, although both the cuticle and gene expression phenotypes are slightly variable. (C) hhts (as in Fig. 1D). (D) ptcIN / Df(2R)44CE; hhts13C Ubx / hhts13C Ubx. The duplication of 1° cell types is absent, there is smooth cuticle posterior to the normally positioned 1° cells, but no 3° cells. Bars are 10 μm.

Dpp does not appear to mediate Hedgehog signaling in the dorsal epidermis

We first re-examined the expression of dpp in the dorsal epidermis to determine if it is expressed in cells adjacent to those expressing hh, as would be expected if Dpp mediated Hh function. Just prior to 6 hours AEL, dpp is expressed globally along the dorsal epidermis (between arrows, Fig. 2A) (Jackson and Hoffmann, 1994). Thereafter, dpp expression decays from dorsal epidermal cells (between arrows, Fig. 2B), remaining expressed only at the edge (Fig. 2B). Note that there are no stripes of dpp expression posterior to hh stripes (arrowhead, Fig. 2B).

We previously showed that cell fates could be respecified through the ectopic expression of hh in transgenic embryos carrying the hh cDNA regulated from a heat shock promoter (HS-hh; Ingham, 1993; Heemskerk and DiNardo, 1994). Brief (5 minutes) or longer (20 minutes) heat-shock treatments caused specific shifts in fates (Fig. 3B and C, respectively). Since in imaginal discs, ectopic hh expression caused ectopic induction of dpp, we investigated whether dpp was induced in HS-hh embryos. In embryos carrying the HS-hh transgene but not heat shocked, dpp expression in the dorsal epidermis is found only at the edge, as expected (Fig. 3E, arrow, and F). However, a relatively long heat shock (40 minutes) caused induction of dpp expression in a stripe of cells dorsally (Fig. 3G). A short heat shock showed no dpp induction, while a 20 minute heat shock showed little if any induction (data not shown). Thus, dpp expression can be induced by ectopic Hh expression, but the induction required longer treatments than that required to cause changes in cell fates.

Nevertheless, to directly test whether ectopic dpp expression can affect cell fates across the dorsal epidermis, we used the GAL4 system to express dpp under the control of GAL4-responsive UAS sequences (Brand and Perrimon, 1993). Embryos containing both HS-GAL4 and UAS-Dpp were given a 40 minute heat shock at 6 hours AEL, and then either fixed 1.5 hours later and processed to verify ectopic Dpp expression (Fig. 3H) or allowed to develop until hatching and analyzed...
for cuticle pattern (Fig. 3D). We found no changes in dorsal pattern across the segment. In addition, the ectopic expression of Dpp driven by ptc-GAL4 also showed no effect on dorsal pattern. We conclude that dpp does not regulate pattern across the segment, and, thus, there must be novel targets for the Hh pathway.

**Hedgehog antagonizes the activity of Patched in the specification of 1° and 2°, but not 3° cell types**

In the absence of Hh activity, only the 4° cell type is specified (Fig. 4C; Heemskerk and DiNardo, 1994). If the loss of 1°, 2° and 3° cell types is due to unchecked activity of ptc, then these cell types should be restored in embryos lacking both Hh and Ptc activity. We find that in such double mutants, the 1° and 2° cell types are again specified (Fig. 4D; Materials and methods). This indicates that the role played by Hh signaling in the specification of these two cell types is to antagonize Ptc activity, as is the case in imaginal patterning and for the ventral epidermis. In contrast to 1° and 2° cell types, the 3° cell types are not restored in ptc, hh double mutants, although there is an occasional thick hair that could represent a 3° cell type (Fig. 4D; Materials and Methods). Thus, 3° cell types are dependent upon Hh activity (Fig. 4C), but hh does not specify these cells by the usual antagonism of the ptc pathway. This suggests that there is a novel target for Hh signaling in the dorsal epidermis.

The 3° cell types are also missing in single ptc mutants (Fig. 4B). This could signify that Ptc activity is required for their specification. However, the 3° cells are replaced by a duplication of large pigmented denticles that are likely to be 1° cell types, with smooth cuticle between the duplicated elements (Fig. 4B; Hooper and Scott, 1989). This duplication of pattern is probably due to the duplication of stripes of en/hh gene expression observed at earlier stages in ptc mutants (DiNardo et al., 1988; Martinez Arias et al., 1988). Thus, the lack of 3° cell types in ptc mutants appears to be a secondary consequence of the earlier changes in gene expression.

**lines function is required for dorsal pattern**

We reasoned that mutations that affected 3° fates might reveal other targets for Hh signaling in the dorsal epidermis. In the absence of lines function, 1° and 2° cell types are established normally, but 4° cell types are completely missing (Fig. 5B). Furthermore, there is an expansion in the number of rows of cells that adopt 3° cell fate from an average of 2.5 to about 7 (Fig. 5, compare A with B). Thus in the absence of lines function, 4° cell types, which are dependent upon wg function, are missing, while 3° cell types, which are dependent upon Hh function, are expanded.

**Hedgehog signaling antagonizes lines function**

Since 3° cell fates are dependent upon Hh activity, we tested whether the expansion of these fates was due to any expansion in Hh influence. First, we found only subtle changes in hh gene expression in lines mutants, and these occurred only after the period during which Hh activity specifies fates (van den Heuvel et al., 1993). However, lines mutations could affect the domain of Hh protein expression, or increase the sensitivity of cells to Hh activity. In both of these cases the specification of 3° cell fates in lines mutants would still be dependent on Hh activity. To test this, we analyzed the cuticle phenotype of lines; hh double mutant embryos. In embryos carrying only the hh<sup>ts</sup> mutation, no 3° cell types are specified if Hh function is removed after 6 hours AEL (Fig. 5C; 1° and 2° cell types are also absent). However, in the lines; hh<sup>ts</sup> double mutant it appears that 3° cell types are restored. The 4° cell types are missing and small denticles similar to 3° cell types are produced (Fig. 5D). Thus, in the absence of lines, 3° cell types are formed independently of hh activity. This implies that hh signaling normally antagonizes the activity of lines and that their regulatory relationship in specifying the 3° cell types is similar to that of hh and ptc in specifying the 1° and 2° fates.

**lines regulates the late expression of Wingless**

We have shown that Wg activity is required between 6-9 hours AEL for 4° cell types (Fig. 1). Thus, the deletion of 4° cell types observed in lines mutants suggests that lines might normally regulate wg expression or function during this interval. In wild-type embryos between 6 to 9 hours AEL, wg is expressed in a patch of cells dorsally within each parasegment (Fig. 6A). As the dorsal epidermis stretches over the amnioserosa the patch becomes a stripe (Fig. 6C). In lines mutants, wg expression is normal through most of the 6-9 hours AEL period (Fig. 6B). It decays prematurely at about 9 hours

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**Fig. 5.** lines activity is required for 4° cell types and to limit the domain of 3° cell types. (A) Wild type (WT). (B) lin<sup>24</sup> / lin<sup>24</sup>. Segments are of approximately normal width in lines mutants, thus there does not appear to be extensive cell death. Cells that normally adopt 4° fates appear to adopt 3° fate in the absence of lines function. (C) hh<sup>ts</sup>. (D) lin<sup>24</sup> / lin<sup>24</sup>; hh<sup>ts</sup>. Shifted to non-permissive temperature (for hh) at 6 hours AEL, 4° cell types are still missing, and are thus dependent on lines function. Similar results are observed in doubly homozygous lin<sup>24</sup>; hh<sup>ts</sup>. Bars are 10 μm.
AEL (van den Heuvel et al., 1993), and expression is absent during dorsal closure (Fig. 6D). We conclude that *lines* activity is required to maintain the expression of *wg*. However, this requirement comes only after the time that *wg* signaling specifies most of 4° cell types (Fig. 1F-H). Thus, the loss of all 4° cell types in *lines* mutants cannot be attributed to loss of *wg* gene expression. It remains possible that *lines* activity is required for Wg signaling. However, the mutant phenotypes are different in that there are extra 3° cell types in *lines* mutants but not in *wg* mutants (compare Fig. 5B with 1F-H). Thus, *lines* must be involved in some other non-Wg-dependent process, or must act independently of Wg signaling.

**DISCUSSION**

We have demonstrated that the Wg and Hh signaling pathways are required for all epidermal pattern dorsally across the segment. Thus, the two rows of cells that flank the parasegment boundary emit signals that are crucial in organizing all segment pattern (Fig. 7A; Heemskerk and DiNardo, 1994). Our results also point to a role for *lines* activity as a cooperating factor in the development of this pattern.

Previous analyses suggested a role for Wg in the dorsal epidermis (Baker, 1988). However, it was thought that Wg acted during a different time interval ventrally as compared to dorsally (Bejsovec and Martinez Arias, 1991). Whereas ventrally Wg signaling was required through 8 or 9 hours AEL, dorsally its activity was only required before approx. 4.5 hours AEL. By examining the pattern of cell-type specification dorsally, we show here that Wg function is in fact required for more than just the previously reported early event. Wg signaling is required from 6 to 9 hours AEL for the 4° cell types. During this same, late period Hh signaling is required for the other cell types (Heemskerk and DiNardo, 1994). Thus, the temporal requirements for Wg and Hh, the two known signals patterning the dorsal epidermis, are identical.

We divide dorsal epidermal patterning into two periods, each with distinct purposes. During the stabilization period signaling occurs between *wg* and *hh*-expressing cells, and this serves to maintain the expression of the other signal (Fig. 7A; Martinez Arias et al., 1988; Cumberledge and Krasnow, 1993; Heemskerk and DiNardo, 1994). During the next period, Wg and Hh activity specifies cell fates (Fig. 7A,B). The best evidence that these two periods serve distinct purposes comes from our analysis of the role of Hh. In embryos where the requirement for early Wg input has been bypassed, Hh activity still appeared to specify the correct cell types dorsally in the absence of any Wg function (Heemskerk and DiNardo, 1994). Therefore early Wg signaling has no role in patterning other than to stabilize the Hh signal, which will pattern later. The question remains open as to whether, reciprocally, there is no other role for early Hh than the stabilization of Wg signaling. No experiment has yet tested whether Wg can execute its patterning role in the complete absence of early as well as late *hh* function.

On the ventral epidermis, it has not been possible to bypass the early requirement of one signal in the maintenance of the other. Thus, it is not known whether there are discrete contributions of Wg or Hh to the specification of particular fates, in the complete absence of the other signal. However, the analysis of single and multiple mutant combinations has led to the
postulate that different combinations of five gene activities, including \(wg\) and \(hh\), are responsible for the diverse ventral cell types across the segment (Bejsovec and Wieschaus, 1993).

There is a strong polarity in signaling for both \(Wg\) and \(Hh\). We find that this extends to the role of \(wg\) in dorsal pattern. In general, during the fate specification period, \(wg\) activity is required in cells to the anterior of \(wg\)-expressing cells (this work; Baker, 1988; Bejsovec and Martinez Arias, 1991). Ventrally, cells adopt the smooth fate, while, dorsally, cells adopt 4\(^{°}\) fate (this work; Wieschaus and Riggleman, 1987; Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992). Reciprocally, \(Hh\) activity is required in cells generally to the posterior of \(hh\)-expressing cells (Heemskerk and DiNardo, 1994). Dorsally, \(hh\) is responsible for cell fates within the \(en\)/\(hh\) domain (1\(^{°}\) cell types), as well as the fates of several rows of cells to the posterior (the 2\(^{°}\) and 3\(^{°}\) cell types). Similarly, \(hh\) is required ventrally for the specification of the posterior \(en\)/\(hh\)-expressing cells, and at least some denticle cell types posterior to this (S.D., unpublished). The molecular basis for the asymmetric requirement for each signal is unclear, although in the case of \(wg\), it appears to be reflected in the asymmetric accumulation to the anterior of steady-state levels of \(Wg\) antigen (van den Heuvel et al., 1989; Gonzalez et al., 1991).

It has been shown that, although \(Wg\) has a strong bias in signaling to the anterior, there is a local signal to the posterior. Ventrally, one row of cells positioned posteriorly to \(wg\)-expressing cells require late \(wg\) input to adopt a smooth fate (Dougan and DiNardo, 1992). There may also be a local, posterior signal dorsally, since in the absence of late \(Wg\) function there appears to be an extra row of 1\(^{°}\) cell types anterior to the normal row (Fig. 1H, arrow).

One broad issue is how \(Wg\) and \(Hh\), produced from narrow strips of cells, influence a larger domain of cells and thereby specify pattern across the segment. The effects of \(wg\) gene function are graded, in that supplying progressively more \(wg\) allows the specification, survival and differentiation of more cells. However, all of these cells are of only one apparent type, fine hairs (4\(^{°}\) cells) dorsally, and smooth cells ventrally. Therefore the graded requirement for \(Wg\) cannot reflect different concentrations of \(Wg\) specifying different fates. Perhaps the progressive requirement reflects the need to achieve some absolute level of activation of the \(Wg\) pathway in a given cell.

In the dorsal epidermis, there is a suggestion that two distinct populations of cells secrete fine hairs. Only a subset of the cells that normally adopt 4\(^{°}\) fates can be transformed into 3\(^{°}\) cell types when exposed to \(Hh\) activity from a heartbeat promoter (Fig. 3C; Heemskerk and DiNardo, 1994). Therefore, there are two domains of 4\(^{°}\) cells, as judged by sensitivity to \(Hh\). In addition, although difficult to score reliably, several rows of fine hairs point posteriorly, while more posterior hairs point anteriorly (Campos-Ortega and Hartenstein, 1985). If a molecular marker were to be identified that removed any ambiguity in scoring this polarity difference, then the issue of whether different concentrations of \(Wg\) were required for the development of these different subdomains could be tested.

In contrast to the role of \(Wg\), \(Hh\) signaling in the epidermis is responsible for several distinct cell fates. We previously provided evidence that signals emanating from \(en\)-expressing cells caused the specification of 1\(^{°}\), 2\(^{°}\) and 3\(^{°}\) cell types in the absence of \(Wg\) function. Thus, there was a \(Wg\)-independent signal (or signals) that specified substantial pattern across the segment dorsally. We showed that \(Hh\) function was required for this pattern, and in particular, that reducing the level of \(Hh\) activity would cause the mis-specification of adjacent cells from their usual fates to a fate normally adopted by cells located more distally to the source of \(hh\)-expressing cells. Lastly, ectopic expression of the \(hh\) gene caused a re-organization of pattern consistent with cells responding to \(Hh\) in a graded manner (Heemskerk and DiNardo, 1994). These results led us to propose that \(Hh\) signaled from \(en\)-expressing cells, that \(Hh\) was solely responsible for the specification of the 1\(^{°}\), 2\(^{°}\) and 3\(^{°}\) cell types, and did so as a morphogen. Recent experiments show that increasing the level of \(Hh\) from the source using the binary GAL4-UAS system had little or no affect on long-range pattern in the epidermis (Fietz et al., 1995). Thus, although \(Hh\) can act as a morphogen, it appears not to do so normally in patterning the dorsal epidermis. As a first step in investigating the graded requirement for \(Hh\) activity that we have demonstrated in the dorsal epidermis, we have begun to investigate the signaling pathways through which and with which \(Hh\) operates.

We find that the specification of 1\(^{°}\) and 2\(^{°}\) cell types requires the antagonism of \(ptc\) activity by \(hh\) (Fig. 4). Thus, for this portion of the dorsal pattern, the regulatory relationship between \(hh\) and \(ptc\) is identical to that in other tissues. It remains to be tested whether the mechanism of action involves inhibition of protein kinase A activity, as has been suggested in other tissues (Fan et al., 1995; Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995).

\(decapentaplegic\) (\(dpp\)) has been implicated as the mediator of the role of \(hh\) in patterning. To test directly whether \(Dpp\) activity similarly mediates the role we have assigned to \(hh\) in patterning the dorsal epidermis, we would need to selectively remove \(dpp\) function late during the fate specification stage. However, there is an earlier requirement for \(Dpp\) activity in establishing global dorsoventral polarity (Irish and Gelbart, 1987; Wharton et al., 1993), and no satisfactory conditional alleles exist that could be used to bypass this early role. Nevertheless, three results suggest that \(dpp\) does not mediate the role of \(hh\) in patterning the dorsal epidermis. Firstly, during the period that \(Hh\) specifies fates, \(dpp\) is not expressed in a position consistent to be a mediator of \(hh\)-induced patterning. Secondly, although \(dpp\) can be induced upon ectopic expression of \(hh\), it appears to require a higher level of ectopic \(hh\) expression to induce \(dpp\) expression than to induce changes in cell fate. Lastly, the direct ectopic expression of \(dpp\) is not sufficient to reorganize cell types across the dorsal epidermis. This contrasts with the wing, leg and eye, where \(dpp\) activity is sufficient to organize pattern (Capdevila and Guerrero, 1994; Fietz et al., 1995; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995; Zecca et al., 1995). It remains possible that a molecule related to \(dpp\) mediates \(hh\) patterning in the dorsal epidermis; however, the two identified family members, \(60A\) and \(screw\), are not expressed in the right place to do so (Wharton et al., 1991; Arora et al., 1994).

The similar regulatory relationships so far observed between \(hh\), \(ptc\) and \(dpp\) concerning the imaginal discs have led to proposals that these signaling systems act as a regulatory cassette to be used in different instances of patterning.
However, in patterning the dorsal epidermis, although hh function antagonizes ptc activity, this does not account for all cell types specified by hh action, since the 3°, hh-dependent cell type is not restored in ptc, hh double mutants. In addition, dpp does not mediate the effects of hh, even though it appears likely that there is some other intermediary to account for the graded response of tissue to hh activity. Thus, our results extend the possible ways through which hh may act, and activity of the gene lines may be one novel target.

There are other roles ascribed to hh where the mechanisms through which it acts are still unclear. For instance, Hh acts over a short range in the wing independently of both Wg and Dpp function (Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995). Although the range is short, it is likely to be several cell diameters, similar to the distance over which hh acts in dorsal patterning. Consequently, we are testing lines in imaginal tissues to see the distance over which it may act. In vertebrates, different concentrations of Sonic hedgehog (Shh) specify either floor plate or motor neuron fate, but no mediators for the action of Shh are known (Roelink et al., 1994; Marti et al., 1995). In addition, during limb patterning, Shh can act as a Zone of Polarizing Activity, and it induces the expression of the dpp homologue, BMP2, in adjacent regions, but experiments that test whether BMP2 might mediate the role of Shh have led to negative results (Francis et al., 1994).

**Patterning the dorsal epidermis**

hh and wg act in two apparently exclusive domains of cells across each segment of the dorsal epidermis. By examining how the different cell types are established we have found that lines plays a significant role at the interface of these two domains, repressing the differentiation of 3° cell types while also promoting the differentiation of 4° cell types. Thus it is important to consider how lines activity might regulate or be regulated by the other two pathways.

lines does not simply regulate wg gene expression. It is also unlikely for lines activity to mediate wg signaling because the wg and lines phenotypes are different. When wg is inactivated cells die since the segment is much shorter than in wild type (Fig. 1F). In lines mutants segment length is the same as wild type (Fig. 5). Thus, in lines mutants, either 4° cells are transformed into 3° cells, or 4° cells die and 3° cells are stimulated to divide. Regardless of the mechanism, the differences between the lines and wg phenotypes suggest that these two activities are independently required for 4° cell survival and differentiation (Fig. 7B). For example, late wg input may be essential for cell viability and lines activity may assign cell identity. Another observation argues for independent roles of Wg and lines. In wg null mutants, where both Wg and Hh signaling is lost early, most cells die, but the few surviving cells differentiate as 4° cells. This appears to be due to lines function, since in lines wg° double mutants these few cells take on 3° rather than 4° fates (unpublished observations).

The role of Hh in patterning involves the selective antagonism of Ptc activity to allow the differentiation of 1° and 2° cell types and the antagonism of lines activity to allow the differentiation of 3° cell types (Fig. 7B). Thus, lines may define a novel pathway under the influence of Hh signaling. These regulatory relationships leave open the question of what molecule(s) specify the fates of the 1°, 2° and 3° cell types, but we have demonstrated that Dpp, an often-used mediator of the role of Hh, appears not to be involved.

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