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Hedgehog acts by distinct gradient and signal relay mechanisms to organise cell type and cell polarity in the *Drosophila* abdomen

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SUMMARY

The epidermis of the adult *Drosophila* abdomen is formed by a chain of anterior (A) and posterior (P) compartments, each segment comprising one A and one P compartment. In the accompanying paper (Struhl et al., 1997), we provide evidence that Hedgehog protein (Hh), being secreted from P compartment cells, organises the pattern and polarity of A compartment cells. Here we test whether Hh acts directly or by a signal relay mechanism. We use mutations in *Protein Kinase A* (*PKA*) or *smoothened* (*smo*) to activate or to block Hh signal transduction in clones of A compartment cells. For cell type, a scalar property, both manipulations cause strictly autonomous transformations: the cells affected are exactly those and only those that are mutant. Hence, we infer that Hh acts directly on A compartment cells to specify the various types of cuticular structures that they differentiate. By contrast, these same manipulations cause non-autonomous effects on cell polarity, a vectorial property. Consequently, we surmise that Hh influences cell polarity indirectly, possibly by inducing other signalling factors. Finally, we present evidence that Hh does not polarise abdominal cells by utilising either Decapentaplegic (Dpp) or Wingless (Wg), the two morphogens through which Hh acts during limb development. We conclude that, in the abdomen, cell type and cell polarity reflect distinct outputs of Hh signalling and propose that these outputs are controlled by separable gradient and signal relay mechanisms.

Key words: Hedgehog, *Drosophila*, segmentation, *smoothened*, gradient, *Protein Kinase A*

INTRODUCTION

The dorsal cuticle formed by each segment of the adult *Drosophila* abdomen derives from two clusters of epidermal cells, the anterior dorsal and posterior dorsal histoblast nests. Although quiescent during larval development, these cells proliferate rapidly in the pupa to generate adjacent but immiscible cell populations that comprise the anterior (A) and posterior (P) compartments of the adult tergite (Madhavan and Madhavan, 1980; Kornberg, 1981). All cells of the posterior nest and their descendents express the genes *engrailed* (*en*) and *hedgehog* (*hh*) (Hama et al., 1990; Struhl et al., 1997). By contrast, no cells that derive from the anterior nest express *en* and this allows them to respond to Hedgehog protein (Hh), which is expressed and secreted exclusively by P compartment cells.

The cells of each A compartment are patterned, the integument varying in pigmentation, hairs and bristles. Also, the epidermal cells are polarised, forming hairs and bristles that point posteriorly. In the accompanying paper (Struhl et al., 1997), we present evidence that Hh spreads across from P compartment cells to form concentration gradients in the neighbouring A compartments. We also make clones of A compartment cells that express Hh ectopically; these alter the pattern and polarity of surrounding wild-type cells in a manner suggesting that both properties are normally organised by Hh.

In principle, Hh could control cell type and polarity by acting as a gradient morphogen. The concentration gradient of Hh could be read like a topographical map, with different threshold concentrations corresponding to contour lines that delimit territories of different cell type. This gradient landscape could also confer vectorial information and polarise cells. For example, cells might be able to 'read' the vector, the direction of maximal change in Hh concentration in their immediate vicinity and respond by aligning structures such as bristles or hairs (Lawrence, 1966, 1992; Stumpf, 1966). Such gradient mechanisms are plausible: there is circumstantial evidence that Hh can function as a morphogen in the dorsal epidermis of the *Drosophila* embryo (Heemskerk and DiNardo, 1994) and in the vertebrate spinal cord (Roelink et al., 1995; Ericson et al., 1996).

Alternatively, Hh might control pattern and polarity in the abdomen by acting indirectly as a short-range inducer of other signalling molecules. This is exactly how Hh exerts a long-range influence on growth and patterning in *Drosophila* limbs, where it acts principally as a short-range inducer of Wingless (Wg) and Decapentaplegic (Dpp), two gradient morphogens (Struhl and Basler, 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994; Jiang and Struhl, 1995; Lepage et al., 1995;

Li et al., 1995; Pan and Rubin, 1995; Zecca et al., 1995; Chen and Struhl, 1996; Lecuit et al., 1996; Nellen et al., 1996; Zecca et al., 1996; reviewed in Lawrence and Struhl, 1996).

To investigate how Hh patterns the adult abdomen, we have performed two types of experiment. First, we have made clones of cells in which the Hh signal transduction pathway is constitutively active and asked whether these cells alter the pattern or polarity of surrounding wild-type cells. If Hh acts directly as a gradient morphogen, such clones should have only autonomous effects. However, if Hh acts indirectly through the induction of other signalling molecules, then these clones should affect surrounding tissue. Second, we have made clones of cells in which the Hh signal transduction pathway is abolished. If Hh acts directly, these mutant cells should be unable to respond to it. However, if Hh acts by inducing other signalling molecules, the response to Hh in surrounding, wild-type tissue should be able to organise pattern and polarity of mutant cells in the clone.

Surprisingly, we have found different answers, depending on whether we assay cell type or cell polarity. We find that abolishing or constitutively activating Hh signal transduction in A compartment clones causes autonomous transformations of cell type, as expected if Hh acts directly as a gradient morphogen. However, such clones have non-autonomous effects on cell polarity, as expected if Hh acts indirectly, through the induction of another factor. Our results also indicate that this factor is neither Dpp nor Wg. We conclude (i) that cell type and cell polarity are controlled by different mechanisms in the developing abdomen, and (ii) that Hh acts in this context both as a gradient morphogen and as an inducer of a polarising signal.

MATERIALS AND METHODS

Induction of marked *PKA* and *smo* mutant clones

Clones of mutant cells were marked variously by loss of the *yellow*$^+$ (y^+) gene, which affects bristle and cuticle colour, by the loss of the *stubby chaete*$^+$ (*stc*$^+$) gene, which affects bristle and hair morphology (Jiang and Struhl, 1995), or by the autonomous up-regulation or loss of expression of the *patched* (*ptc*) gene (Struhl et al., 1997). All clones were generated by Flp-mediated mitotic recombination (Golic, 1991), using proximally located Flp Recombination Targets (FRT's) described previously (Chou and Perrimon, 1992; Xu and Rubin, 1993). The genotypes used for each experiment are listed below, and references are given to the relevant figures. Further information about the genotypes used can be found elsewhere (Jiang and Struhl, 1995; Chen and Struhl, 1996; Struhl et al., 1997). In general, clones were induced by heat shock (34°-36°C for 60 minutes) during or soon after the blastoderm stage to eliminate the wild-type gene prior to the allocation of cells to the dorsal histoblast nests. Clones were identified and prepared for staining and/or microscopy as described in Struhl et al. (1997).

PKA mutant clones

PKA$^-$ clones marked by *y* and *stc* (Figs 2, 5): *y hsp70-flp.1/y* or *Y*; *PKA*$^-$ *stc FRT39 /CD2L.1, y*$^+$ *FRT39.*

PKA$^-$ clones marked by *stc* in a y^+ background (Figs 3, 4C): *y hsp70-flp.1/+; PKA*$^-$ *stc FRT39 /CD2L.1, y*$^+$ *FRT39.*

PKA$^-$ clones marked by *stc* and *y* in a *ptc-lacZ* background (Figs 4A,B): *y hsp70-flp.1/y* or *Y*; *PKA*$^-$ *stc FRT39 /CD2L.1, y*$^+$ *FRT39; ptc-Z(III)/+*.

PKA$^-$ clones marked by *y* in a *ptc-lacZ* background (Fig. 8, 10): *y hsp70-flp.1/y* or *Y*; *PKA*$^-$ *stc FRT39 ptc*^{AT96}*/CD2L.1, y*$^+$ *FRT39.*

wg$^-$ *PKA*$^-$ clones marked by *y* and *stc* (Fig. 9): *y hsp70-flp.1/y* or *Y*; *wg*^{CX4} *PKA*$^-$ *stc FRT39 /CD2L.1, y*$^+$ *FRT39.*

smo mutant clones

smo$^-$ clones marked by *stc* in a y^+ background (Fig. 6B): *y hsp70-flp.1/+; smo*^{3} *stc FRT39 /CD2L.1, y*$^+$ *FRT39.*

smo$^-$ clones marked by *y* in a *ptc-lacZ* background (Figs 6A, 7, 11B): *y hsp70-flp.1/y* or *Y; smo*^{3} *FRT40 ptc*^{AT96}*/Dpsc*^{19}, y^+ *FRT40.*

smo$^-$ clones marked by *y* and *stc* in a *ptc-lacZ* background (Fig. 11A): *y hsp70-flp.1/y* or *Y; smo*^{3} *stc FRT39 /CD2L.1, y*$^+$ *FRT39; ptc-Z(III)/+*.

RESULTS

During limb development in *Drosophila*, the Hh signal transduction pathway can be constitutively activated within cells by removing Protein Kinase A (PKA), a component which, in the absence of Hh, blocks the pathway (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995). Conversely, the ability of cells to transduce Hh depends on the transmembrane protein Smoothened (Smo) (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996). Here, we describe the phenotypes associated with somatic clones of PKA or Smo deficient cells generated by Flp-mediated mitotic recombination (Golic, 1991). Mutant cells were marked variously with the *yellow* (*y*) and *stubby chaete* (*stc*) mutations and by the expression of *ptc-lacZ*, which indicates the state of activity of the Hh pathway (Struhl et al., 1997), so that the genotype, as well as the type and polarity, of virtually every cell could be scored unequivocally. As described in the accompanying paper (Struhl et al., 1997), cells in the anterior and posterior parts of the A compartment are exposed to opposite gradients of Hh secreted, respectively, by the P compartments fore and aft. We begin first with the effects of mutant clones in the posterior portion, where cell type and polarity can be easily scored, and then extend our analysis to the anterior part.

Autonomous transformations of cell type caused by activating or blocking the Hh transduction pathway

(i) Activating the Hh pathway (*PKA* mutant clones)

The caudal region of a typical A compartment shows a posterior-to-anterior progression of four types of cuticle: a6 (unpigmented), a5 (darkly pigmented with large bristles), a4 (darkly pigmented with smaller bristles) and a3 (lightly pigmented with smaller bristles) (Struhl et al., 1997). This pattern correlates with an inferred gradient of Hh protein. We find that *ptc-lacZ* expression is maximal in a6 cells, and declines progressively in a5 and a4 cells, falling beneath the level of detection in a3 cells.

Clones of cells homozygous for *DCO*^{E95}, an apparent null allele of the *DCO* locus, which encodes PKA (Li et al., 1995; Jiang and Struhl, 1995, referred to subsequently as *PKA*$^-$), develop normally or almost normally when they arise in the A compartment near to the A/P compartment boundary, the region that forms a5 and a6 cuticle (Figs 1, 2). Although mutant cells do not alter the a6 cuticle and still form normally positioned a5 bristles, they often lack pigment; that is there appears to be a transformation of a5 towards a6 cuticle (Fig. 3) This is not surprising, as the most posterior cells are normally exposed to high levels of Hh spreading from the P

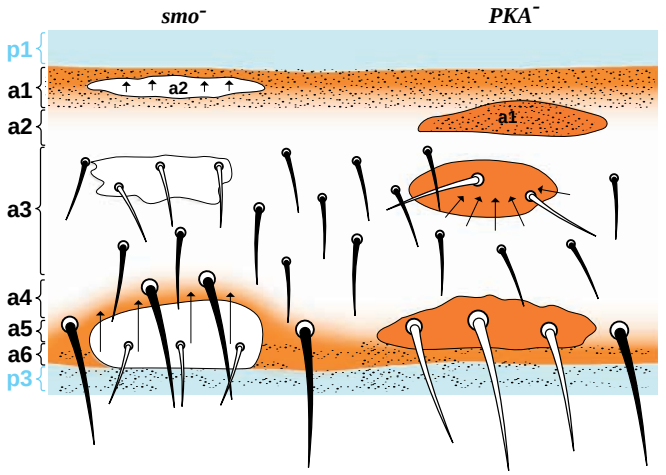

Fig. 1. Summary of the phenotypes of *smo* and *PKA* mutant clones. A typical abdominal tergite is shown: P compartments (shown in blue) are positioned immediately fore and aft of the A compartment. Hh secreted by P compartment cells spreads into the A compartment across both the anterior and posterior edges to form opposing concentration gradients; these gradients can be monitored by the expression of a *ptc-lacZ* gene (shown in red) which is induced by Hh. Cells in the A compartment form a progression of distinct cuticle types (a1,a2,a3,a4,a5 and a6; see text and Fig. 4 in Struhl et al., 1997) distinguished by pigmentation and adornments (e.g., hairs, bristles), all of the latter having a common polarity, pointing posteriorly. *smo* (left) and *PKA* (right) mutant clones are shown marked by y^- (white bristles); *smo* and *PKA* mutant clones were also marked, respectively, by the absence, or constitutive high level of expression, of *ptc-lacZ* (shown as white or red within the clone boundaries). Bristles characteristic of a5 cuticle are larger than those of a4 and a3 cuticle: as diagrammed, bristles formed by *smo* mutant clones are invariably of the a3/a4 type, whereas those formed by *PKA*$^-$ clones are typically of a5 type. *smo* mutant clones replace a1 cuticle with a2 cuticle, while *PKA* mutant clones replace a2 cuticle with a1 cuticle. Hair polarity is normal unless arrows indicate otherwise.

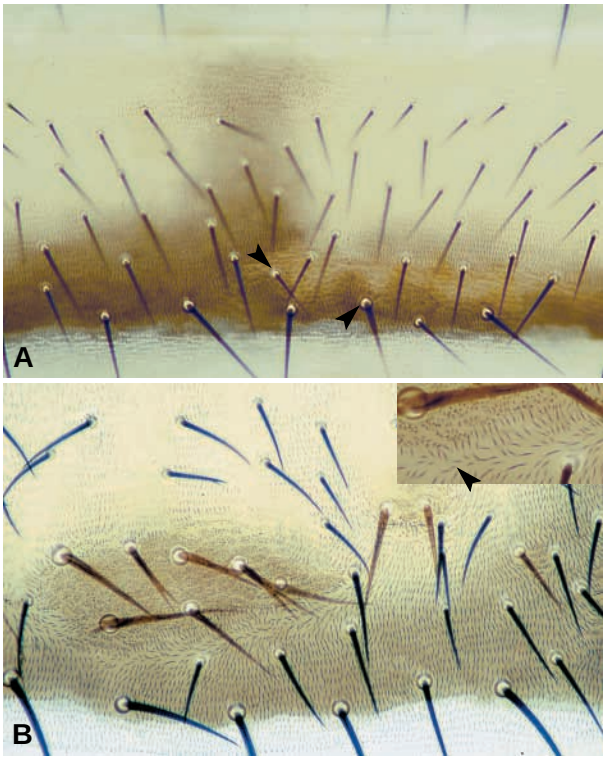

Fig. 2. *PKA*$^-$ clones: formation of a5/a6 cuticle. (A) *PKA* mutant clone marked with y^- and *stc*$^-$ in the a5 region of the A compartment: Note the two y^- bristles (arrowheads), and wiggly stripe of yellowish cuticle which marks the clone. All of the hairs secreted by the mutant cells are split into tufts by the *stc* mutation: the effect of this marker can be seen at higher magnification in B. Aside from the markers, the clone appears phenotypically normal. (B) Clone in the a3/a4 region. Note that the clone appears split into two islands of mutant tissue: posterior to each island, hairs have reversed polarity (see inset). Posterior to the hairs having reversed polarity, there is a zone of hairs that point laterally or even upwards (arrowhead), and further posteriorly the hairs have normal polarity. y^- bristles within the clonal patches are larger than typical a3 bristles and resemble those of a5 cuticle; they also tend to be crowded. See Materials and Methods for exact genotypes.

compartment, with a6 regions receiving more Hh than a5. If the loss of PKA activity elevates, but does not maximally activate, the Hh signal pathway, then endogenous Hh from the P compartment might 'raise' the level of mutant cells in the a5 region from a5 towards a6. In the wing, loss of PKA activity also appears to elevate, but not maximally activate, the Hh pathway (Jiang and Struhl, 1995).

PKA$^-$ clones that arise more anteriorly, in the a4 and a3 regions, are abnormal in at least three respects: First, the clones show elevated levels of *ptc-lacZ* expression, and this is strictly autonomous to the clone (Fig. 4). Second, these *PKA*$^-$ clones form a5-type cuticle, as indicated by their dusky pigmentation and by the larger size of the bristles (Fig. 3); this transformation is also cell-autonomous (Fig. 3). Third, the hairs and bristles formed by *PKA*$^-$ cells are more crowded than surrounding wild-type tissue, an effect that increases the more anterior the clone is; such crowded clones have a less wiggly boundary and tend to be circular (Fig. 4C). Anterior *PKA*$^-$ clones also appear to be under-represented: indeed, we observe some clones in which the main mass of marked cells is posterior in the A compartment, but from which thin fingers of marked cells reach forward (Fig. 5, see legend). We suspect that the main mass of such clones, which is a5 in type, originated further anteriorly but has moved back to be nearer to the a5 cells of the host. Grafting experiments on other insects have shown that cells taken from similar levels in the A/P axis of a segment can intermingle while those from different levels sort out from each other, suggesting a gradient of cell affinity along the A/P axis (Wright and Lawrence, 1981). Hence, we interpret the crowding, sorting out behaviour and apparent backwards migration of parts of *PKA*$^-$ clones as due to their having been respecified as a5-type tissue and having gained a5-like affinities.

Thus, *PKA*$^-$ clones in the abdomen affect cell type, the effects being exactly limited to the cells of the clone. They differ from ectopic Hh-expressing clones, which reorganise the pattern of cell types in surrounding, wild-type territory (Struhl et al., 1997). We interpret these results as evidence that, in the abdomen, Hh specifies cell type — a scalar property — by acting directly on responding cells, and not by proxy through the induction of other factors.

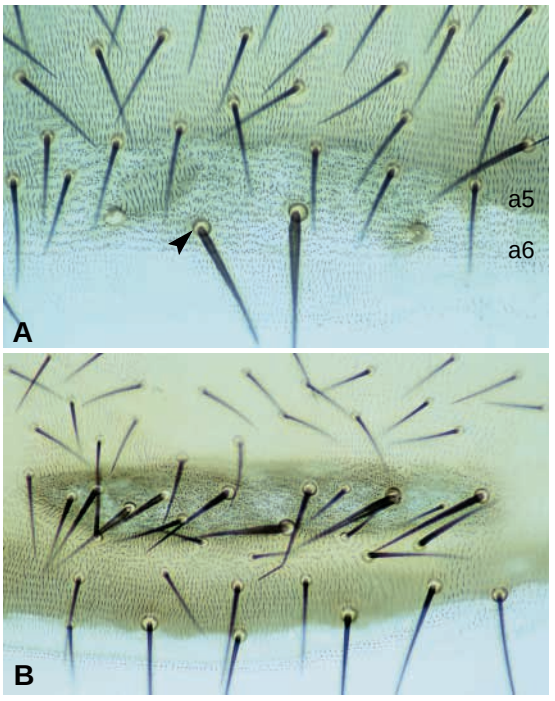

Fig. 3. Autonomous effects of *PKA*$^-$ clones on pigmentation. (A) *PKA*$^-$ clone marked only with *stc*$^-$ (i.e., in a *y*$^+$ background) situated at the extreme posterior edge of the A compartment, in the a6 and a5 territories. It is not strongly pigmented, that is it makes cuticle of the a6 pigmentation even in the a5 region. The two large a5 bristles shown are *PKA*$^-$ and marked with *stc*$^-$ (one is arrowed). (B) *PKA*$^-$ *stc*$^-$ clone in the a3 region. Note that the clone has the dusky pigment and the large bristles typical of a5 cuticle. There is some light mottling of this, to make small patches of unpigmented (a6) cuticle. There is a barely detectable spread from the clone of dusky pigment, about one cell in diameter and we think this is due to spread of the pigment itself or some precursor; supporting this interpretation we have found that *PKA*$^-$ *y*$^-$ *stc*$^-$ clones in the a3 region do not show a halo of dusky pigment around them, as would be expected if they caused surrounding cells to form a4 or a5 cuticle.

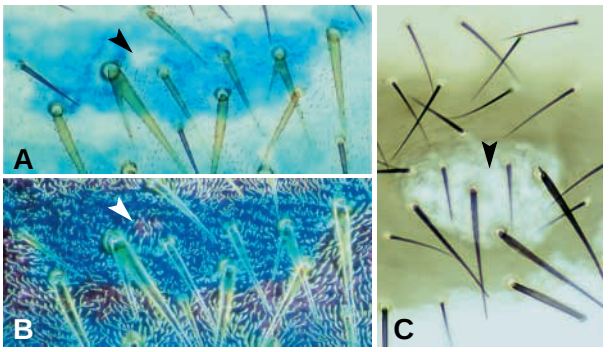

Fig. 4. Autonomous effects of *PKA*$^-$ clones on gene expression and cell sorting. (A,B) Bright- and dark-field views of a small *PKA*$^-$ clone marked with *y*$^-$ and *stc*$^-$ in a *ptc-lacZ* background. The upper picture (bright-field) shows the clone staining in blue because *ptc* is up-regulated. There is a small patch of one or two cells that is not stained blue (arrowhead). The bristles within the clone are *y*$^-$ and of the size of a5 bristles. The lower picture (dark-field) shows that the blue patch and the *stc* territory are coextensive, and that the small hole in the blue patch shown above coincides with a few *stc*$^+$ hairs (arrowhead). (C) *PKA*$^-$ clone marked only with *stc*$^-$ in the A6 segment (both mutant and wild-type cells are *y*$^+$). Note that the clone (arrowed), which makes the slightly pigmented cuticle that is normally found just behind the dark pigment in that segment as well as some larger bristles, becomes circular as it sorts out to some extent from the background. The clone is also raised above the surround.

Fig. 5. Evidence for posterior movement of *PKA* mutant cells. A large *PKA*$^-$ clone marked with *y*$^-$ and *stc*$^-$. It appears to extend from the extreme anterior region, where a2 cuticle is replaced by a1 cuticle, that is smooth and unpigmented (arrowhead), via a thin trail of marked cells to join with the main mass of the clone that is transformed from a3 into a5 cuticle (note the large a5-type *y*$^-$ bristles). We speculate that many of the cells have moved posteriorly to associate with *PKA*$^+$ cells of a5 type, leaving the trail behind.

(ii) Blocking Hh signal transduction (*smo* mutant clones)

Hh is received by an unusual mechanism in which the serpentine transmembrane protein Smoothened (Smo) and the multiple-pass transmembrane protein Patched (Ptc) have distinct functions (Chen and Struhl, 1996; Marigo et al., 1996; Stone et al., 1996). Cells lacking Smo activity behave as if they cannot transduce Hh (Hooper, 1994; Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996). In addition, they differ from wild-type cells in being unable to sequester Hh. As a consequence, Hh appears to spread through *smo* mutant clones until it reaches and is transduced by wild-type cells on the other side of the clone (see Chen and Struhl, 1996).

Clones homozygous for the *smo*^{3} mutation, which appears to abolish Smo activity (Chen and Struhl, 1996), develop normally when they arise in the middle of the A compartment, in the region forming a3 cuticle (Fig. 6A). This result is expected as these cells are furthest from the P compartments and are exposed to little if any Hh (Struhl et al., 1997). By contrast, *smo*^{3} clones that arise near or next to the A/P boundary are transformed; they make a3-type cuticle instead of the normal a4, a5 or a6 cuticle (Figs 7, 8). Moreover, they do not up-regulate *ptc-lacZ* expression (Fig. 7). By both these criteria the transformation is autonomous to the clones (Figs 7,

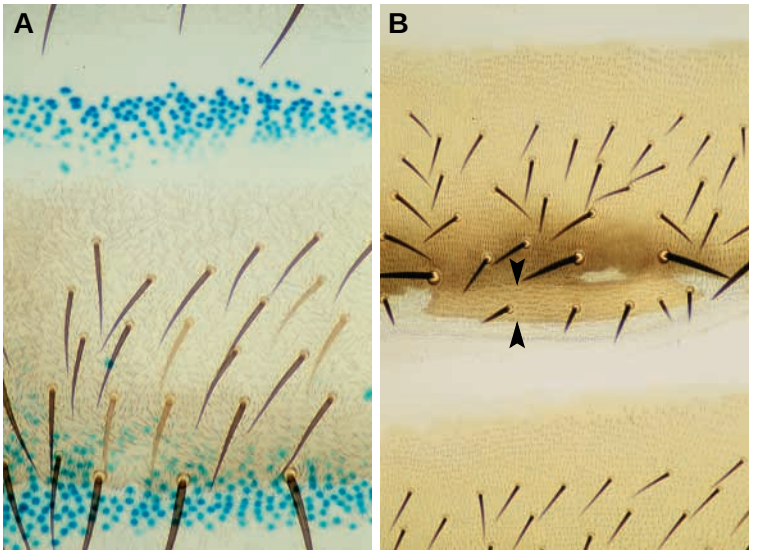

Fig. 6. *smo* mutant clones: formation of a3-type cuticle. (A) Large *smo* mutant clone marked with y^- in a *ptc-lacZ* background. It is located centrally in the a3 region of the A compartment and develops normally. (B) *smo* mutant clone marked with stc^- in a y^+ background: the clone (between arrowheads) is located in the a6 region which normally lacks pigment. It is lightly pigmented and forms moderate sized bristles, like a3 cuticle, but lacks the dusky colour characteristic of a5 and a4. This clone illustrates the transformation from a6 to a3-type cuticle. The discerning reader will note that the fly is carrying the *Stubble* mutation and that there is a second *smo* mutant clone adjacent to the first, but in the P compartment.

8). This autonomy is all the more striking because clones of smo^3 cells that abut the A/P compartment boundary are associated with ectopic Hh signalling immediately anterior to the clone. We presume that Hh has spread unimpeded through the clone and been received on the other side by cells that respond by expressing *ptc-lacZ* and forming a5 cuticle. Consequently, these smo^3 clones appear as islands of a3 cuticle bounded posteriorly by the P compartment and anteriorly by ectopic a6 and a5 cuticle (Fig. 7).

Thus, smo^3 cells form a3 cuticle irrespective of whether they are exposed to Hh, or are adjacent to wild-type A compartment cells, which are receiving and responding to Hh. This finding reinforces our conclusion, based on the *PKA* mutant clones, that abdominal cell type is specified directly by Hh.

Non-autonomous effects on cell polarity

(i) *PKA* mutant clones

In the wild type, all cells that secrete a6, a5, a4 and a3 cuticle form hairs or bristles which point posteriorly, that is towards the P compartment of the same segment, the nearest source of Hh. Clones of PKA^- cells that arise close to the A/P compartment boundary, in the a6 and a5 region, do not alter the polarity of these structures either within the clone or in neighbouring wild-type tissue (summarised in Fig. 1). However, PKA^- clones that arise more anteriorly, in the a3 and a4 region, alter the polarity of hairs and bristles, both within the clone and outside it. The effect on surrounding cells can be seen most clearly in the case of PKA^- clones that are marked with the *stc* mutation that distinguishes mutant from wild-type hairs with single cell resolution (Fig. 2B, inset). In general, wild-type cells positioned laterally and posteriorly to the mutant clone form hairs and bristles that point centripetally towards the clone (Figs 3B, 8); thus, behind the clone, cells form hairs and bristles that point anteriorly. The region of wild-type tissue showing this 'reversed' polarity can be up to 4 cell diameters wide. Still further behind the clone there is a thin strip of cells that exhibit

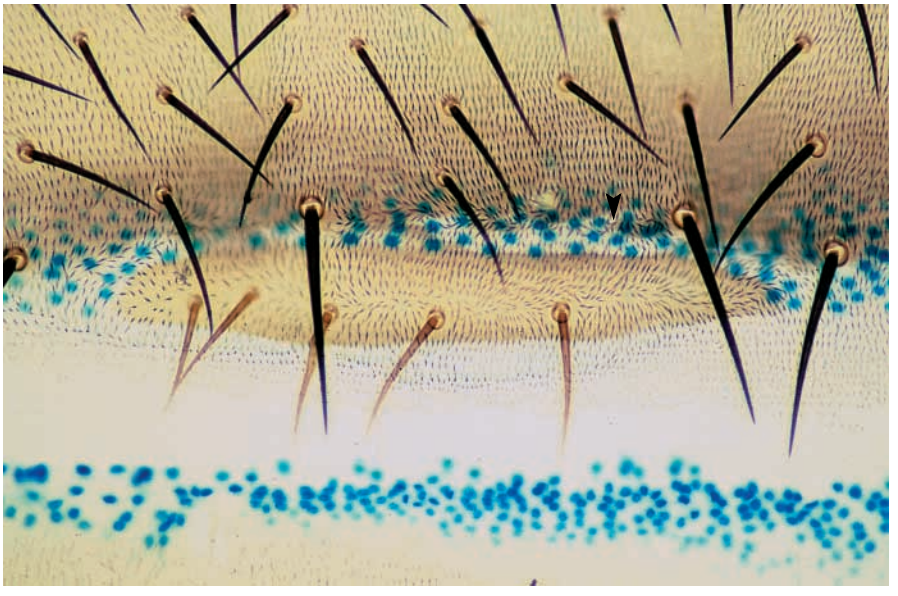

Fig. 7. Non-autonomous effects of *smo* mutant clones on hair polarity. smo^- clone marked with y^-, but not with stc^-, in a *ptc-lacZ* background: the clone is at the extreme posterior limit of the A compartment and develops a3 pigmentation. Within such islands of *smo* mutant tissue, we usually find a3-type bristles just posterior to the middle of the clone, often arranged in a mediolateral line and pointing posteriorly, as shown also in Fig. 6B. Note that the clone excludes *ptc-lacZ* expression and also that there is no *ptc-lacZ* expression immediately posterior to it. In the anterior two thirds of the clone, as well as just anterior to the clone, the hairs point forwards, while hairs in the posterior third point backwards, like the bristles. In a narrow zone of wild-type cells, situated two or three cell rows anterior to the clone, the hairs have random or even upwards polarity (arrowhead).

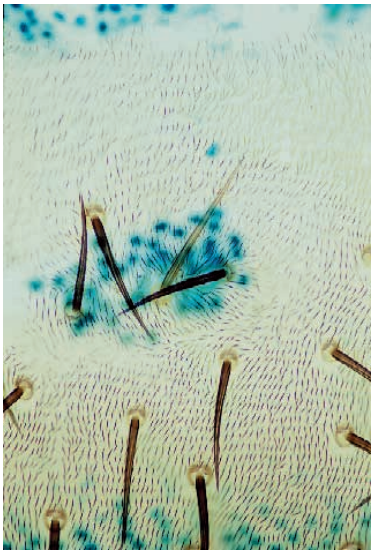

Fig. 8. Centripetal hair polarities associated with a *PKA* mutant clone in the a3 region. *PKA*$^-$ clone marked with *y*$^-$, but not with *stc*$^-$, in a *ptc-lacZ* background: *ptc-lacZ* expression is autonomous to *PKA* mutant cells (Fig. 4A,B) and hence the central patch of blue nuclei marks the mutant cells. Note that the orientation of the hairs in the clone is centripetal, with those in the more posterior part pointing anteriorly. There is also a single *y*$^-$ bristle that points anteriorly.

neutral polarity (they form hairs and bristles that appear to point at random and sometimes directly upwards, that is orthogonal to the plane of the epidermis). Non-autonomous effects on polarity also occur when Hh is expressed in the same region of the segment (Struhl et al., 1997). However, unlike ectopic Hh-expressing cells, *PKA*$^-$ mutant clones transform cell type and up-regulate *ptc-lacZ* expression in a strictly cell autonomous fashion, indicating that they are not serving as ectopic sources of Hh.

These findings suggest that Hh normally polarises surrounding wild-type cells through an indirect mechanism, that is, through another factor. If this were true, one might expect the factor to be induced and distributed uniformly in a *PKA*$^-$ clone, giving no cue of polarity to cells within the clone. However, we find that the cells both within and surrounding *PKA*$^-$ clones in the a3 region make hairs and bristles that tend to point towards the centre of the clone; indeed, hairs and bristles in the back portion of the clone have 'reversed' polarity — they point anteriorly (Fig. 8). One possibility is that the loss of PKA activity induces the synthesis of a secreted factor that can polarise cells both within and outside of the clone; centrifugal diffusion of this factor might then form a graded concentration landscape with a peak within the clone and altered slopes extending outside of it.

(ii) *smo* mutant clones

The properties of *smo* mutant clones provide two further tests of whether Hh acts directly or indirectly to polarise A compartment cells.

First, as described above, *smo*^{3} clones in the middle of the A compartment make a3 cuticle, which we interpret as the default 'off' state when no Hh signal is received. However, as shown in Fig. 6A, all the bristles and hairs secreted by these *smo*^{3} cells point posteriorly. Hence, even though these cells cannot themselves respond directly to Hh, they nevertheless are correctly polarised, as if responding to signals generated by wild-type cells positioned closer to the A/P compartment boundary which have received and transduced Hh.

Second, A compartment clones of *smo*^{3} cells that arise along the A/P compartment boundary cannot receive Hh, yet most of the cells belonging to these clones form structures that have reversed polarity, pointing anteriorly towards the ectopic *ptc-lacZ*-expressing *smo*$^+$ cells in front of the clone. This reversal in polarity also extends out into about two cell rows of wild-type cells; in front of these there is a line of cells with neutral polarity and, further anteriorly, they show a return to normal polarity (Fig. 7).

Thus, the alterations in cell polarity associated with *smo*^{3} and *PKA*$^-$ clones yield a consistent picture. In the case of *smo*^{3} clones, the mutant cells are exposed to Hh, but incapable of receiving it. Nevertheless, they form hairs and bristles that point towards the nearest *smo*$^+$ cells that are transducing Hh. In the case of *PKA*$^-$ clones, the surrounding wild-type cells are capable of receiving Hh, but are not exposed to Hh secreted by the mutant cells. Yet, they form hairs and bristles that point towards the mutant cells that are the nearest in which the Hh pathway is active. Hence, cells do not appear to be polarized by the direct action of Hh, but rather, by other factors induced in response to Hh.

There is an exception to this general rule: although most of the hairs in *smo*^{3} clones that abut the A/P compartment boundary have reversed polarity, the hairs formed by mutant cells close to the boundary have normal polarity (Fig. 7). We are reasonably confident that the normal polarity of these cells is not a response to any remaining wild-type cells that are responding to Hh, because such cells should express *ptc-lacZ* — no such cells were seen (Fig. 7). One possible explanation is that P compartment cells might secrete a Hh-independent polarising factor, which acts locally on A compartment cells just across the A/P boundary (see Discussion). Another is that *smo*^{3} cells along the boundary are showing a residual response to *smo*$^+$ larval cells that were able to respond to Hh. Such larval cells do not persist to the adult.

The polarising signal: evidence against Wg and Dpp

Hh organises growth and patterning in the limbs by inducing either *wg* or *dpp* and therefore it might act in the abdomen through one or both of these genes. Consistent with this idea, *dpp* and *wg* are expressed in stripes of anterior cells positioned adjacent and anterior to the A/P compartment boundary within each abdominal segment, *wg* in the tergites and sternites, and *dpp* in the ventral pleura (Shirras and Couso, 1996; Struhl et al., 1997).

If Hh works through Wg to influence polarity, removal of *wg* from clones of cells that are activated in the Hh pathway should eliminate that influence. We made this test and compared *wg*$^-$ *PKA*$^-$ *stc*$^-$ clones with *PKA*$^-$ *stc*$^-$ clones: we found no difference between them, in both cases clones in the a3 region form a5 cuticle and alter the polarity of neighbouring wild-type cells (compare Figs 8 and 9). Thus, neither the change in cell type nor the alterations in cell polarity caused by the loss of *PKA*$^-$ activity appear to be due to the ectopic expression of *wg*.

In the legs, Wg signalling blocks expression of *dpp* in response to Hh; hence the simultaneous elimination of both *wg* and *PKA* leads to ectopic expression of *dpp*, which in turn can reorganise limb pattern (Jiang and Struhl, 1995; 1996; Brook and Cohen, 1996; Theisen et al., 1996). To test for this, we made *dpp*$^-$ *wg*$^-$ *PKA*$^-$ *stc*$^-$ clones, but could not distinguish the phenotype of these from either *PKA*$^-$ *stc*$^-$ or *wg*$^-$ *PKA*$^-$ *stc*$^-$ clones (data not shown), arguing against a role for Dpp.

Fig. 9. *wg*$^{-}$ *PKA*$^{-}$ clone associated with reversed hair polarity in neighbouring wild-type tissue. *wg*$^{-}$*PKA*$^{-}$ clone marked with *y*$^{-}$ and *stc*$^{-}$: note that posterior to the mutant territory, the wild-type hairs have reversed polarity, as observed for *wg*$^{+}$ *PKA*$^{-}$ clones (compare with Fig. 3B). The mutant cells are also transformed from a3 towards a5, a marked a5 bristle is arrowed.

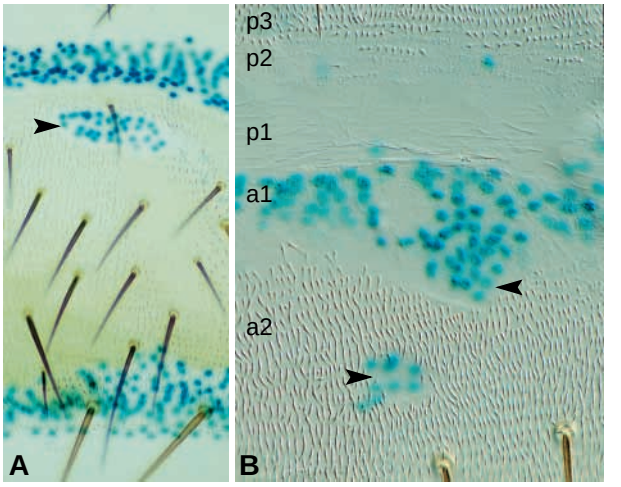

Fig. 10. a2-to-a1 transformations in cell type caused by *PKA*$^{-}$ clones in the anterior portion of the A compartment. (A) A *PKA*$^{-}$ mutant clone marked by the constitutive expression of *ptc-lacZ* (arrowed) forms a1 (smooth) cuticle instead of a2 (hairy) cuticle. Astute readers may note a *y*$^{-}$ clone in the a5 region. (B) Two patches of *PKA*$^{-}$ mutant cells, marked as in A, are present close to the anterior edge of the A compartment (arrowheads). Both show autonomous transformation from a2 into a1 cuticle.

Thus, the polarising activity associated with the loss of PKA activity does not appear to be either Wg or Dpp.

Effects of *PKA* and *smo* mutant clones in the anterior portion of the A compartment

Based on the pattern of *ptc-lacZ* expression in wild-type animals, we infer that Hh normally enters the A compartment across the anterior, as well as the posterior edge, to form two concentration gradients (Struhl et al., 1997). At the peak of the anterior gradient, cells make a1 cuticle, while at a lower concentration of Hh and further into the compartment, they make a2 cuticle (Struhl et al., 1997). In a2 cuticle, the cells form hairs that point 'down' the anterior Hh gradient, unlike cells in the posterior half of the compartment, which point 'up' the opposing Hh gradient (see Fig. 14 in Struhl et al., 1997).

We find that *PKA*$^{-}$ and *smo*^{3} mutant clones in the anterior region of the A compartment alter cell type much as they do in the posterior portion. For example, *PKA*$^{-}$ clones in the a2 region (marked by ectopic *ptc-lacZ* expression) are transformed to make naked a1 cuticle, that is they act as if they are receiving more Hh (Fig. 10), whereas *smo*^{3} clones in the a1 region autonomously differentiate hairs (as in a2 cuticle), that is, as if they are receiving little or no Hh. Moreover, some clones of *smo*^{3} cells in the a1 region form hairs that have reversed polarity (Fig. 11) and these hairs point away from the source of the ectopic polarising signal, which we infer is positioned posterior to the clone. These observations support the conclusion (Struhl et al., 1997) that cells within the anterior and posterior portions of the A compartment are programmed to respond in different ways to Hh.

Finally we note that, even though *PKA*$^{-}$ clones tend to make either a1 or a5 cuticle, depending on whether they arise anteriorly or posteriorly, all clones cannot be classified into one class or the other. Single *PKA*$^{-}$ clones can span much of the A compartment, with anterior cells forming a1 cuticle and posterior ones forming a5 tissue (Fig. 5), suggesting that there is no early lineage restriction between the anterior and the posterior subpopulations.

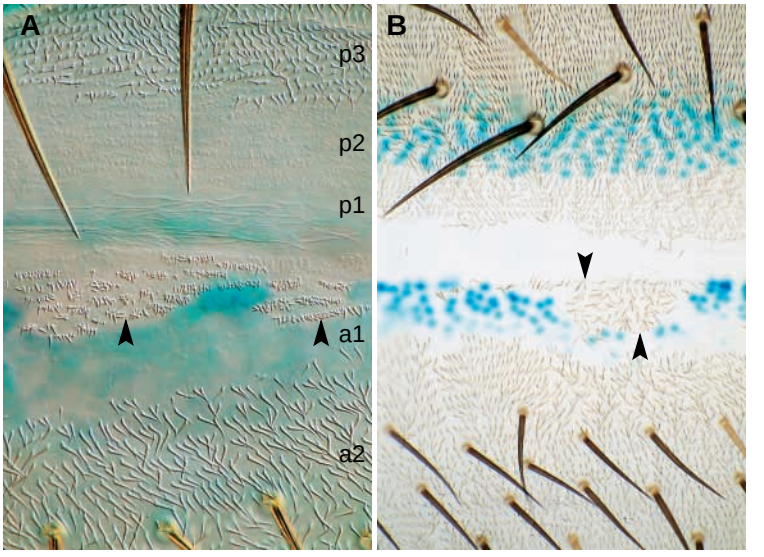

Fig. 11. a1-to-a2 transformations in cell type caused by *smo* mutant clones in the anterior portion of the A compartment. (A) *smo* mutant clone marked with *stc*$^{-}$ (arrowheads) in a *ptc-lacZ* (cytoplasmic) background. The clone is in the a1 region, but autonomously differentiates as a2 cuticle (hairs, no bristles) and also excludes *ptc-lacZ* expression. The p3, p2, p1, a1 and a2 cuticles are indicated on the right. (B) *smo*$^{-}$ clone marked with *y*$^{-}$, but not *stc*$^{-}$, in a *ptc-lacZ* background. The clone of mutant cells (between arrowheads), does not express *ptc-lacZ*, and abuts the anterior edge of the A compartment. It makes a2 cuticle with hairs. At the lower arrowhead, there is a line of *ptc-lacZ*-expressing cells behind the clone and, just anterior to this line, the hairs have reversed polarity, pointing forwards.

DISCUSSION

Studies of segmental patterns in insects have shown that cell type and cell polarity are organised by signals emanating from the vicinity of the boundaries between segments. These signals can direct cells at defined distances from the boundaries to differentiate as particular cell types (e.g., to secrete a distinctive ridge or to synthesise a pigment). In addition, they can polarise cells to make oriented cuticular structures such as folds or hairs. These properties were interpreted as evidence for a gradient mechanism in which cell type and cell polarity are specified by the concentration (scalar) and slope (vector) of a diffusible morphogen spreading from one segment boundary to the next (reviewed in Struhl et al., 1997).

These earlier researches were carried out before it was known that segments are divided into A and P compartments and before any genes engaged in generating positional information were identified. In the accompanying paper, we apply this newer knowledge to the adult *Drosophila* abdomen and present evidence that Hh protein secreted by P compartment cells controls both the pattern and polarity of A compartment cells (Struhl et al., 1997). Here we ask how Hh works: does it act directly as a gradient morphogen, or does it affect pattern largely via signal relay, as is the case in the *Drosophila* limb (reviewed in Lawrence and Struhl, 1996)? Unexpectedly, we find that Hh employs both mechanisms but uses them for different outputs, functioning as a gradient morphogen to specify cell type and as an inducer of other signals to polarise cells.

Control of abdominal cell type by Hh: a gradient mechanism

During normal development of the abdomen, Hh is secreted only by P compartment cells, yet it has a long-range influence on the neighbouring A compartments. In principle, Hh could act directly or indirectly. To distinguish between these alternatives, we make clones of cells in which the Hh signal transduction pathway is abolished or constitutively activated. Such clones are expected to show a strictly cell autonomous response if Hh acts directly, but non-autonomous effects if Hh operates by a signal relay system.

In the abdomen, we find that both gain and loss of the activity of the Hh pathway cause autonomous transformations in cell type and these transformations are exactly those expected if Hh were to function as a gradient morphogen with high points at both anterior and posterior limits of each A compartment, that is with a U-shaped distribution. And indeed, Hh appears to form a U-shaped landscape of opposing gradients (Struhl et al., 1997). We conclude that, at least with regard to cell type, Hh acts as a true morphogen in this tissue.

Our present findings contrast with the results of equivalent studies of Hh-signalling performed in the developing limbs. In particular, experiments in which the Hh signal transduction pathway is blocked or constitutively activated have established that Dpp and Wg mediate the long-range organizing activity of Hh during *Drosophila* wing and leg development (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Chen and Struhl, 1996). However, there is evidence that Hh does function directly, albeit at shorter range, to control some aspects of patterning in the limbs (e.g. Jiang and Struhl, 1995; Zecca et al., 1995; Gómez-Skarmeta and Modolell, 1996; Mullor et al., 1997). Hence, Hh may operate even here, as in the abdominal epidermis, by a gradient mechanism. This possibility is supported by limited evidence that different concentrations of Hh can elicit distinct outcomes both in the embryo and in the developing limbs (Heemskerk and DiNardo, 1994; Ingham and Fietz, 1995; but see also Fietz et al., 1995; Bokor and DiNardo, 1996); more compelling evidence has recently been reported in studies of patterning in the vertebrate neural tube (Roelink et al., 1995; Ericson et al., 1996).

Equivalent results to those presented here for Hh have recently been obtained for Dpp and Wg — evidence that these other signalling molecules can function as morphogens (Lecuit et al., 1996; Nellen et al., 1996; Zecca et al., 1996). However, our present studies differ in one significant respect. For both Dpp and Wg, the effects of manipulating signal transduction were assayed by monitoring the transcription of target genes such as *optomotor blind*, *spalt*, *vestigial* and *Distalless*. All of these target genes encode transcription factors, and therefore they could mediate the organising activities of Dpp and Wg, not directly but by driving the expression of other secreted factors. If this were the case, the pattern would be finalised by signal relay systems (discussed in Nellen et al., 1996; Zecca et al., 1996). Our present results are not subject to this qualification because we monitor the final pattern itself. Hence, they support the conclusion that different concentrations of a morphogen (such as Hh) can be read out directly as a progression of distinct cell types, as proposed in general terms long ago (Lawrence, 1966; Stumpf, 1966, 1968; Wolpert, 1969).

How might Hh specify cell type? Consider a4 and a3 cuticle in a middle abdominal segment: they differ only in pigmentation, having dusky and light pigmentation respectively, apparently because they derive from cells that have received different amounts of Hh. Hh signalling regulates the level, and possibly the state of activity or subcellular localisation, of Cubitus interruptus (Ci), a transcription factor that is instrumental in the Hh pathway (Alexandre et al., 1996; Dominguez et al., 1996; Hepker et al., 1997). Hence, a gradient of extracellular Hh might yield a graded distribution of active Ci within cells. We suggest that Ci drives transcription of genes involved in pigment synthesis (e.g., those encoding tanning enzymes) when present above a critical threshold level, leading to the dusky pigmentation that distinguishes a4 from a3 tissue. As we note in the accompanying paper (Struhl et al., 1997), the particular cell types specified by given concentrations of Hh vary from segment to segment, depending on the activity of the BX-C complex. Like Ci, the principal products of the BX-C are transcription factors. Hence BX-C proteins may act directly and together with Ci to drive the patterned expression of particular target genes (e.g., the dusky pigmentation genes) in specific segments.

Control of abdominal cell polarity by Hh: a signal-relay mechanism

Although Hh appears to organize the polarity of cuticular structures formed by A compartment cells, our results indicate that it does so indirectly. First, we find that *PKA* mutant cells alter the polarity of surrounding wild-type cells as if they were secreting ectopic Hh. Yet, these surrounding cells do not appear to be receiving any ectopic Hh because they neither express

ptc-lacZ (a sensitive assay for Hh) nor secrete types of cuticle that are specified by Hh. Second, we find that the polarity of *smo* mutant cells responds to Hh signalling, even though they cannot transduce Hh. Our evidence suggests that this response depends on secondary signals induced by Hh in neighbouring smo^+ cells.

How might Hh polarise cells via a signal-relay mechanism? One clue is that within and surrounding some *PKA* mutant clones the hairs and bristles point inwards, towards the centre. A simple model is that the loss of PKA activity in these cells mimics reception of Hh and hence induces them to secrete a diffusible polarising factor, 'X'. Because mutant cells in the centre of the clone would be surrounded by X-secreting cells, they might be exposed to higher levels of X than mutant cells at the periphery. As a consequence, X would accumulate in a bell-shaped distribution, peaking at the centre of the clone and declining outwards and into the surrounding wild-type tissue. If cells were oriented by the direction of maximal change (the vector) in the concentration of X, cells both inside and outside of the clone would point towards the centre of the clone, as we observe.

Although such a model fits with our results, it presents several problems. First, cells would have to be able to detect the direction of maximal change in the concentration of X, and, as discussed elsewhere (Lawrence and Struhl, 1996), this is a demanding condition, especially as cells are polarised consistently over many cell diameters. It might be easier if Hh signalling were to act at short range to initiate the expression of X, which could then affect neighbouring cells and activate expression of X in those cells; the result would be a propagating front. A similar model has been put forward to explain planar cell polarity in the *Drosophila* eye (Heberlein et al., 1995; Wehrli and Tomlinson, 1995). Such a propagation model does not demand that X be diffusible, because polarity could be organised by local cell-cell interactions, which spread as in a game of dominoes. In this case, an initial asymmetry in Hh signalling would initiate the cascade. However, this extreme form of the propagation model does not fit easily with our finding that cells both within and outside of PKA mutant clones form structures that point centripetally. Nor does it satisfactorily account for the continuous and smooth whorl-like patterns that are associated with Hh-expressing (Struhl et al., 1997, Fig. 12) or PKA^- clones (Fig. 8), or after grafting experiments in other insects.

The second problem with models in which a gradient or propagating wave of X polarises cells is that all cells within the A compartment form structures with a common polarity, the hairs and bristles all pointing posteriorly. Nevertheless, we have concluded that Hh forms a U-shaped landscape and, therefore, that hairs and bristles in the anterior portion of each A compartment must point 'down' the Hh (or 'X') gradient while, in the posterior portion, they point 'up' the opposing gradient (Struhl et al., 1997). This interpretation is supported by our finding that *smo* mutant clones have opposite effects on hair polarities in the anterior and posterior portions of the A compartment, presumably because cells in these regions respond in opposite ways to the signals produced by ectopic Hh signalling. Although we have suggested that these differing responses may be due to the activity of factors such as Sloppy-paired in posteriorly, but not anteriorly, situated cells, we recognise that this explanation lacks direct experimental support and is unsatisfactory in other ways. In particular, it is difficult to understand how the opposing gradients of X could be coextensive with the cell populations programmed to respond in opposite ways; yet such registration must occur if all the cells are to secrete hairs that point the same way (Struhl et al., 1997). An alternative possibility is that Hh might induce posteriorly and anteriorly situated cells to express different factors, e.g., X posteriorly and Y anteriorly, which polarize cells in opposite directions.

A third problem is that cells in both compartments form hairs and bristles that show a consistent polarity, which extends without deviation across the A/P compartment boundary. If, as we suggest above, Hh induces a diffusible polarizing factor X as it crosses from the P into the A compartment, then one might expect this factor to accumulate in a bell-shaped distribution centered at a position anterior to, rather than coincident with, the A/P compartment boundary. Such a distribution might generate polarity reversals just anterior to the compartment boundary and, indeed, we observe exactly this kind of response just anterior to *smo* mutant clones, which create an ectopic boundary of Hh-responding cells within the A compartment (Fig. 7). The fact that such polarity reversals do not occur along the normal A/P compartment boundary raises the possibility that P compartment cells serve as the source of one or more additional signals capable of polarizing A compartment cells. The existence of such signals would also explain the behaviour of cells at the posterior edges of *smo* mutant clones that abut the P compartment (Fig. 7): these cells form hairs and bristles of normal polarity even though they cannot transduce Hh and are unlikely to be exposed to a posterior source of factor X.

A final problem is that our present studies apply only to polarity in the A compartment. We think it likely that interactions between A and P compartment cells are also responsible for generating the factors responsible for polarizing P compartment cells. However, we do not know whether the same or similar factors are involved, or how their activities are related to the factors controlling cell polarity in the A compartment.

The nature of the polarising factor 'X'

An important unknown is the nature of the signal or signals that polarise cells under the control of Hh. In the developing limbs, it is clear that Hh acts indirectly through Dpp and Wg. However, we have tested whether either of these proteins is responsible for polarising abdominal cells downstream of Hh and found that neither is essential. This conclusion is not so surprising in the case of Dpp, as it is not expressed in cells giving rise to the abdominal tergites. However the *wg* gene is transcribed in A compartment cells in a graded pattern that peaks posteriorly at the A/P compartment boundary and declines progressively in more anterior cells (Shirras and Couso, 1996) — consistent with a role in polarising these cells.

One possibility is that Hh might induce expression of *wg* and also one or more related *Wnt* genes, such as *DWnt2*, *DWnt3* or *DWnt4*, any or all of which could have polarising activity. To test for this, it would be necessary to eliminate *Wnt* genes as well as *wg* from *PKA* mutant clones and see if the polarising activity were abolished. We note that mutations in *frizzled* or *dishevelled* disturb cell polarity in the abdomen (Gubb and Garcia-Bellido, 1982) and that both genes are implicated in Wg/Wnt signal transduction (Noordermeer et al., 1994; Theisen et al., 1994; Bhanot et al., 1996). Hence, it remains

possible that Wnts are directly involved in cell polarisation, despite our initial negative result with Wg.

CONCLUDING REMARKS

Our findings point to what may be a general phenomenon in pattern formation. Hh acts in at least two ways: it can organise cells by acting directly as a morphogen, or by proxy through the induction of other signals. The strategy followed may depend on the effective range of the molecule. For example, Hh acts directly, but in a relatively local domain, to specify the a1, a4, a5 and a6 types of abdominal cuticle instead of the a2 and a3 types of cuticle that form by default in the absence of Hh signalling. By contrast, Hh acts indirectly, but over a more extended domain including the regions forming a2 and a3 types of cuticle, to polarise cells. The wing is significantly larger than an abdominal tergite and forms a more complex pattern; perhaps for this reason, Hh operates through the induction of a second morphogen (Dpp in this case) to extend its realm of influence. In larger insects than *Drosophila*, it might be that secondary morphogens are needed even in the abdomen. For example, *Rhodnius*, *Oncopeltus* and *Dysdercus*, the insects used in the earlier studies of patterning, have abdominal segments that are, at maturity, many times the size of those of *Drosophila*. So although we imagine that Hh plays a similar role in these segments, it may depend on additional morphogens to specify the full repertoire of cell types.

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