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The Hedgehog morphogen and gradients of cell affinity in the abdomen of *Drosophila*

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SUMMARY

The adult abdomen of *Drosophila* is a chain of anterior (A) and posterior (P) compartments. The *engrailed* gene is active in all P compartments and selects the P state. Hedgehog enters each A compartment across both its anterior and posterior edges; within A its concentration confers positional information. The A compartments are subdivided into an anterior and a posterior domain that each make different cell types in response to Hedgehog. We have studied the relationship between Hedgehog, *engrailed* and cell affinity. We made twin clones and measured the shape, size and displacement of the experimental clone, relative to its control twin. We varied the perceived level of Hedgehog in the experimental clone and find that, if this level is different from the surround, the clone fails to grow normally, rounds up and sometimes sorts out completely, becoming separated from the epithelium. Also, clones are displaced towards cells that are more like themselves: for example groups of cells in the middle of the A compartment that are persuaded to differentiate as if they were at the posterior limit of A, move posteriorly. Similarly, clones in the anterior domain of the A compartment that are forced to differentiate as if they were at the anterior limit of A, move anteriorly. Quantitation of these measures and the direction of displacement indicate that there is a U-shaped gradient of affinity in the A compartment that correlates with the U-shaped landscape of Hedgehog concentration. Since affinity changes are autonomous to the clone we believe that, normally, each cell's affinity is a direct response to Hedgehog. By removing *engrailed* in clones we show that A and P cells also differ in affinity from each other, in a manner that appears independent of Hedgehog. Within the P compartment we found some evidence for a U-shaped gradient of affinity, but this cannot be due to Hedgehog which does not act in the P compartment.

Key words: *engrailed*, *patched*, *smoothened*, Compartments, Affinity gradients

INTRODUCTION

When cells of different type are dissociated and mixed they do not adhere to each other at random; rather they tend to form clumps with their own kind (Townes and Holtfreter, 1955). This behaviour is due to a cell-autonomous property which has been called affinity (Garcia-Bellido, 1966, 1972; Holtfreter, 1939). Cadherins are a class of proteins responsible at least in part for cell affinities (Takeichi, 1990). For example: L cells normally contain little or no cadherins but they can be made to express them artificially. It is found that two populations of L cells which differ only in whether they express E or P cadherins, 'sort out' from each other forming largely separate populations (Nose et al., 1988). In the *Drosophila* ovary, there is evidence that the oocyte settles where the concentration of cadherin in the follicle cells is highest (Godt and Tepass, 1998; González-Reyes and St. Johnston, 1998).

Steinberg (1963) proposed the differential adhesiveness model; he treated cells as analogous to molecules in a liquid: they are mobile yet limited in their movements by mutual adhesion. When two cell populations of different affinity are mixed they assume a configuration that minimises free energy. This would mean that, in two dimensions, the more adhesive groups of cells take up circular shapes, surrounded by the less adhesive ones (Garrod and Steinberg, 1973). The model also predicts that cells expressing the same adhesion molecule but differing in amount would sort out from each other, which they do (Friedlander et al., 1989; Steinberg and Takeichi, 1994).

Nardi and Kafatos (1976a,b) studied these phenomena in the moth wing, and transplanted squares of cells along the proximodistal axis. If the transplants were removed and simply replaced they remained square and kept their size, but if they were transplanted along the axis, they became rounded and small; the further the translocation, the more severe the effect. Using the arguments and model of Steinberg, Nardi and Kafatos concluded that there is a proximodistal gradient of adhesiveness in the moth wing.

Like the wing, each insect segment is subdivided into an anterior (A) and a posterior (P) compartment (see accompanying paper, Lawrence et al., 1999). Clones of cells

normally have a wiggly border, showing that dividing cells mingle within a compartment. But clones have a straight edge at the compartment boundary, suggesting that A and P cells have different affinities (Morata and Lawrence, 1975). But even within one compartment there is evidence for spatial variations in affinity: Locke (1959) made transplants of epidermal cells in the abdomen of an insect and concluded there is a gradient of cell affinity in the anteroposterior axis. Since then several criteria for assessing affinity have been proposed. Nübler-Jung (1974) argued that when grafts are made between areas of different affinity, they contract, giving a higher cell density in the graft. Wright and Lawrence (1981) proposed that affinity differences can determine the straightness of the boundary between populations of cells. They found that when two groups of cells deriving from the same level in the anteroposterior axis meet they form and maintain a wiggly interface between them. However, when cells from disparate positions in the anteroposterior axis are brought together they make a straight interface. Wright and Lawrence proposed that there is a gradual change of affinities across the segment with an abrupt change at the compartment boundary.

In *Drosophila* another criterion to study affinity has been used: this is the displacement of the progeny of one marked cell with respect to the clone made by the descendants of its sister, its 'twin' (Ripoll et al., 1988). Twin clones can be made in which one sister clone lacks *smoothened* (*smo*) a gene essential for a response to Hedgehog protein (Hh; Ingham, 1998) and the other is normal, apart from a marker. If these twins are generated in the posterior region of the anterior (A) compartment, the *smo*$^{-}$ clone frequently moves back and into territory normally occupied by posterior (P) compartment cells, leaving its twin in A territory. This 'sorting back' may imply that the cells of the *smo*$^{-}$ clone, which no longer see Hh, have more affinity with P than with the nearby A cells (Blair and Ralston, 1997; Rodríguez and Basler, 1997; discussed in Lawrence, 1997).

In this paper we use several of the above criteria to study cell affinities in the adult abdomen of *Drosophila*. We conclude:

1. That the cells of the P compartment, as a consequence of *engrailed* (*en*) expression therein, have affinities that are distinct from A cells.

2. In addition and independently of the above, we find that affinities are graded along the anteroposterior axes of both A and P compartments.

3. In the two domains of the A, and possibly also in two domains in the P, the gradients of affinities have opposing orientations.

4. The two gradients of affinity in the A compartment appear to be direct readouts of the level of Hh – the factor(s) determining pattern and affinity in the P compartments is unknown.

MATERIALS AND METHODS

Mutations, insertions and transgenes

The FlyBase (http://gin.ebi.ac.uk:7081/) entries of the mutations, insertions and transgenes referred in the text are as follows:

ptc^{-}: *ptc*^{IIw}, an amorphic allele of the *patched* gene.

en^{-}: *Df(2R)en*^{E}, a deletion for both the *invected* and *engrailed* genes.

smo^{-}: *smo*^{3}, an amorphic allele of the *smoothened* gene.

Psmo^{+}: an insertion of *smo*$^{+t6.2}$ in the 2R carrying the whole *smo* gene which rescues the *smo*$^{-}$ phenotype.

hs.FLP: *FLP1*$^{hs.PS}$, *S. cerevisiae* FLIP recombinase under the control of the *hsp70* promoter.

en.FLP: *FLP1*^{en}, FLIP recombinase under the control of the en promoter (an approx. 8 kb fragment of the *en* gene ending about 30 bp before the ATG was inserted in place of the *hsp70* promoter in the *hs.FLP* transgene)

FRT42: *P{ry[+t7.2]=neoFRT}42D.*

CD2y^{+}: *CD2*$^{hs.PJ}$.

Mutant clones

To induce marked clones using *hs.FLP* (see Lawrence et al., 1999) flies of the following genotypes were heat shocked for 30 minutes to 1 hour at 31 or 33°C at different stages of development as stated in the text:

- *hs.FLP/+; smo*$^{-}$ *b FRT42 cn sha/FRT42 pwn ptc*$^{-}$.
- *hs.FLP/+; smo*$^{-}$ *b FRT42 cn sha/FRT42 pwn en*$^{-}$.
- *hs.FLP/+; smo*$^{-}$ *b FRT42 cn sha/FRT42 pwn ptc*$^{-}$ *en*$^{-}$.
- *hs.FLP/+; smo*$^{-}$ *b FRT42 cn sha/FRT42 pwn.*
- *hs.FLP/+; smo*$^{-}$ *b FRT42 cn sha/smo*$^{-}$ *FRT42 pwn Psmo*$^{+}$ *en*$^{-}$.

To induce marked clones only in the P compartment, flies of the following genotypes were used:

- *y w/+; en.FLP FRT42 cn sha/FRT42 pwn.*
- *y w; en.FLP FRT42 cn sha/FRT42 pwn en*$^{-}$ *CD2y*$^{+}$.
- *y w; smo*$^{-}$ *FRT42 sha Psmo*$^{+}$*/smo*$^{-}$ *en.FLP FRT42 pwn en*$^{-}$ *CD2y*$^{+}$.

Measurements and statistics

Abdominal cuticles were prepared as before (Struhl et al., 1997a). The perimeters of the clones as well as the positions of the a1/a2 and a5/a6 borders were traced with the help of a camera lucida and a graphic tablet into NIH Image v. 1.59 installed in a PowerBook 165c. The area (A), perimeter (L) and centroid of each clone were measured. A measure of the shape of the clones ($4\pi A/L^2$) was used; this gives 1.00 for a circle – the more irregular the shape, the lower the value.

To measure the positions of *pwn*$^{-}$ clones with respect to their *sha*$^{-}$ twins, the difference between y_{pwn} and y_{sha} was calculated (Fig. 1) and *t*-tests for paired comparisons (Sokal and Rohlf, 1995) were carried out (A clones: n=27; P clones: n=24). The same tests were performed to determine if the shape and area of the *pwn*$^{-}$ clones differ from their *sha*$^{-}$ twins.

Each clone was mapped by the position of its centroid relative to the two reference borders (as a percentage). A Welch's approximate *t*-test (Sokal and Rohlf, 1995) was used to determine if the distribution of clones induced between 0 and 2 hours after puparium formation (n=30) differed from those induced at later stages (12-14 hours, n=58).

To determine if there is a correlation between the shape of the *ptc*$^{-}$ *en*$^{-}$ clones, area and/or position in the A compartment, we measured 30 clones at 0-2, 40 at 4-6, 57 at 8-10 and 58 at 12-14 hours after puparium formation.

To compare the survival of *smo*$^{-}$ *en*$^{-}$ and *en*$^{-}$ clones in the P compartment, we set up matched experiments and heat shocked them at the end of the larval period. Ten flies carrying clones were then taken from each experiment and the positions of the clones classified by eye as being in the anterior, middle or posterior parts of the P compartments. The *en*$^{-}$ clones were almost entirely confined to the anterior region (17/18); the *smo*$^{-}$ *en*$^{-}$ clones were found in all three regions; anterior (14), middle (13) and posterior (10).

RESULTS

Experimental design

The abdomen consists of a chain of alternating A and P

compartments: *en* is the selector gene responsible for the P state; therefore if this gene (as well as its sister gene, *invected*) is removed from a P cell, that cell divides and forms a clone which differentiates with A character (see Lawrence et al., 1999). We can therefore study the effects of any differences of cell affinity between A and P cells by looking at the behaviour of en^- cells (now-A) in the P compartment.

The organisation of the A compartments in each segment of the abdomen depends on Hh spreading in from the flanking P compartments, to form a U-shaped landscape of concentration that provides positional information to the cells (Struhl et al., 1997a,b). The A compartment is subdivided into a smaller anterior domain of a1 and a2 cuticle, and a larger posterior domain of a3-a6 cuticle (see Fig. 1 in Lawrence et al., 1999). Hh has different effects in the two domains: in the anterior domain the highest concentration specifies a1, while in the posterior domain the highest concentration specifies a6 (Lawrence et al., 1999; Struhl et al., 1997b). Cells in the middle of the A compartments, remote from the sources of Hh, form either a2 or a3 cuticle, depending on whether they belong to the anterior or posterior domains, respectively.

A cell's measurement of the Hh signal utilises receptors and other proteins; clones of cells which lack one these proteins will receive inappropriate positional information. Accordingly, cells from the posterior domain that cannot respond to Hh (because, being smo^-, they lack a component of the receptor) will develop as if they were remote from a source of Hh. Thus clones of a3 cells can be made near the posterior limit of the A compartments, amongst a5 and a6 cells. The opposite experiment can also be done. For example, if the Hh signalling pathway is activated in presumptive a3 cells in the middle of the segment by removing the *patched* (*ptc*) gene, the cells develop as if they were near the border of the A compartment, making a6 cuticle instead (Lawrence et al., 1999). Both these experiments are reminiscent of grafting experiments where patches of cells were moved up or down the segment (Locke, 1959).

In most experiments we have labelled both the mutant clone of interest and the (otherwise wild type) sister clone. The crosses are constructed so that, following mitotic recombination, each of the twin clones carries a different marker; we have used *pawn* (*pwn*) and *shavenoid* (*sha*), mutations that alter the cuticle without affecting other aspects of development and pattern. For studying the effect of ptc^- we have also made ptc^- en^- clones; this genotype avoids a difficulty: if clones that either express Hh or maximally stimulate the pathway (ptc^-) are made in the anterior part of the A compartment, *en* is activated in the clone, transforming the cells into P, with complex results (Lawrence et al., 1999; Struhl et al., 1997b). This problem is circumvented by removing *ptc* and *en* at once.

Our main results are depicted in Fig. 1.

Twin clones in the A compartment

(i) Control twin clones in the A compartment: *pwn/sha*

These clones are wild type except for the markers. We have compared the *pwn* and the *sha* twins by three different criteria. These are, size (an estimate of the rate of net growth), circularity (a measure of the shape of the clone, and an estimate of the affinity difference between the clone itself and the cells surrounding it) and relative position of the clones (is there any directed migration of one of the twins relative to its sister?). For all these measures we find no significant differences ($P>>0.05$; see Fig. 1 and Methods) in these controls, when we compare each twin with its sister. A typical twin spot is shown in Fig. 2A, note the highly wiggly boundaries of both clones.

(ii) Twin clones in the A compartment: *pwn* ptc^- en^-/*sha*

The anterior domain of A consists of a1 and a2 cuticle – here, in the ptc^- en^- clones, the a2 cuticle is transformed to a1 (Lawrence et al., 1999). The posterior domain includes all the a3, a4, a5 and a6 cuticle – here, ptc^- en^- clones, wherever they are located, make a5 cuticle. Heat shocks were given at different times in the early pupae, when the histoblasts are dividing rapidly (Madhavan and Schneiderman, 1977). Following heat shocks given later than 12 hours after puparium formation (APF) the clones are small and the phenotype mixed, due to the variable perdurance of ptc^+ and en^+. But, when induced before this time the mutant clones show that they have different affinities from the cells around them: they have a more circular shape ($P<<0.001$; we found 25/27 cases in which the mutant clone was more circular than its wild-type twin) and are significantly smaller ($P<<0.0005$), averaging about one half the size of their twins. They appear to have a somewhat higher cell density than the surrounding cells.

We asked whether affinities or other properties of the cells might be graded or polarised, and found six pieces of evidence.

1. In the posterior domain of the A compartment, the later the induction, the further anterior ptc^- en^- clones can be found

We consider those ptc^- en^- clones in the posterior domain of the A compartment (a3-a6). When ptc^- en^-/*sha* twins are induced in the larval period, there are many large control *sha* clones which are so big and so ubiquitous they cannot be individually mapped. However, their ptc^- en^- sisters are smaller and mainly located at the back of the domain (in one set of flies 66/78 ptc^- en^- clones were located in the posterior half, and most of these 66 were in the posterior quarter). We believe the more anterior clones disappear because they sort out from the epidermis, forming separated vesicles of cuticle that can be seen in the haemolymph. There is a significant correlation between the time of induction of the clones and their survival in the anteroposterior axis: the later the clones are induced, the further anterior they are found ($P\approx0.001$). Sometimes, usually at the anterior limit of their range, the clones are caught partially sorted out, surviving as vesicles clinging to the cuticle by narrow necks. The smallest clones (made after about 12 hours APF) show less complete transformation and many survive, with their twins, all over the A compartment. Such clones are only a few cells in size. We conclude that ptc^- en^- clones tend to sort out, and the more anterior, the more readily they sort.

2. In the posterior domain of the A compartment, the more anterior the ptc^- en^- clone, the more circular it is

There is a correlation between the shape of the clone and its position. In clones induced at 8-10 hours APF, the shape correlates significantly with position ($P<0.001$), being more circular in the anterior region. At stages earlier than this there

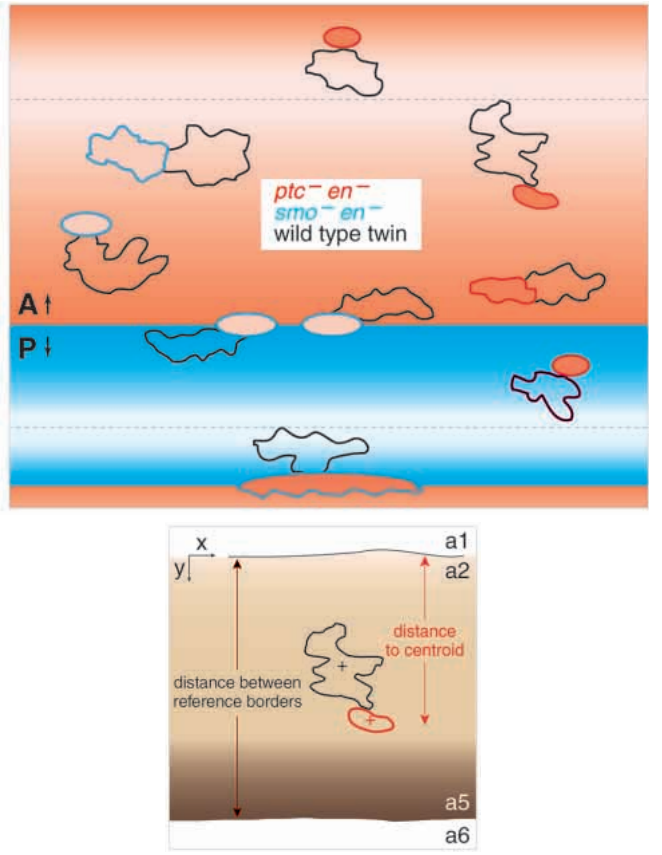

Fig. 1. The measurements carried out on the clones. Above, we show an idealised collection of clones as a vade-mecum. The U-shaped variation in affinity of both compartments is stylised in brown (A) and blue (P). The dashed lines subdivide each compartment, approximately into two domains. Note the genotype, phenotype, shape and relative position of the twins. Below we show the outline of a real case, the *sha* clone is shown in black with its *pwn* twin in red; the crosses mark the centroids, used in measurement of position and orientation.

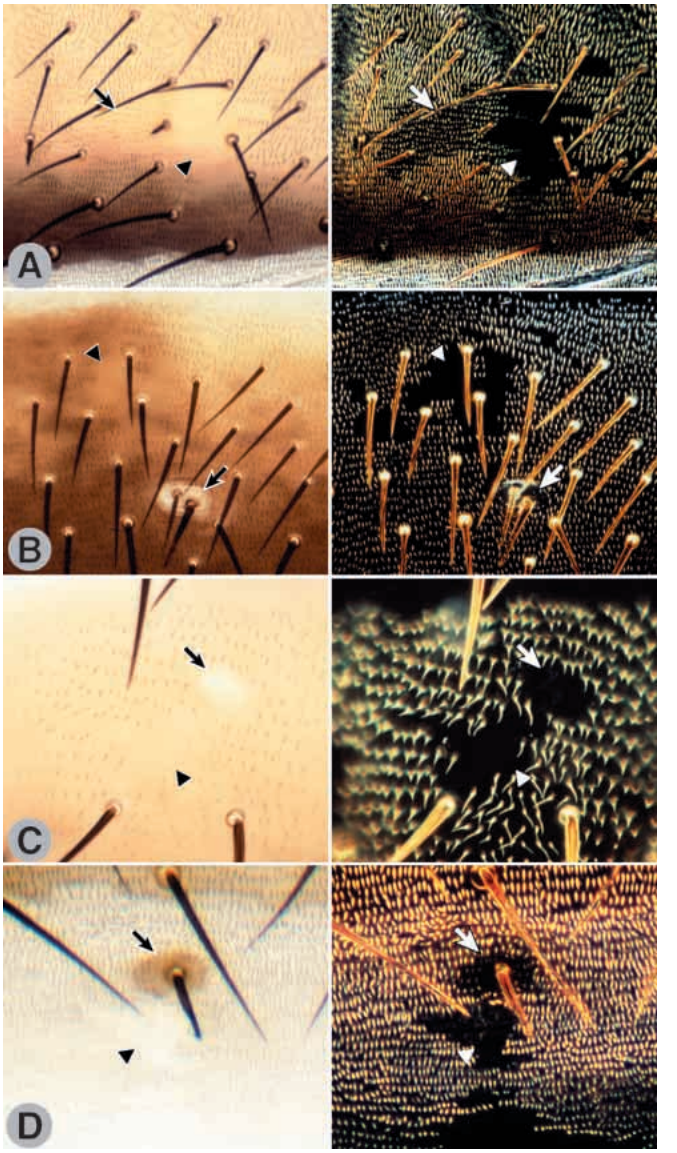

Fig. 2. Twin clones show relative migration of sisters. Twin clones, in which one sister cell, usually also carrying other mutations, is labelled with *pwn* (which mutilates bristles and hairs) and the other is labelled with *sha* (which removes hairs). The left hand column shows the twins in bright field, to show pigmentation, and the right hand column in dark field to show hairs. (A) Control, both sister cells are wild type, except for the markers; note both *pwn* (arrows) and *sha* (arrowheads) clones have wiggly boundaries; each contains about 50 cells. (B-D) The *pwn* clones are mutant for either *ptc* alone (B), or *ptc* and *en* (C), or *en* (D), their sisters are wild type apart from the *sha* marker. (B) The twin clones are of a3 origin and were induced in a young pupa; note that the *pwn* clone is posterior to its twin and is much smaller and more circular in shape, it differentiates unpigmented cuticle of the a6 type. (C) The twin clones are of a2 origin and were induced in a young pupa; note that the *pwn* clone is anterior to its twin and is smaller and more circular in shape, it differentiates unpigmented cuticle of the a1 type. (D) The twin clones are of posterior origin and the small and circular *pwn* twin (which makes a5 cuticle) is found anterior to its *sha* sister.

is no significant correlation, probably because the surviving clones are nearly all confined to the posterior extreme of the A compartment and all are relatively circular in shape. Following heat shocks at later stages the clones show no such correlation, but in these cases the clones have only a partial phenotype and all the clones are unevenly shaped.

3. In the posterior domain of the A compartment, the more anterior the *ptc⁻ en⁻* clone, the smaller it is

The growth of the mutant clones was surprising: when clones are induced at different times during the first 10 hours APF one would expect the clones to become smaller and smaller with later and later heat shocks, and this is true of the *sha* twins. However, the average area of the mutant clones changes rather little with time of heat shock, suggesting that the expansion of clones induced early is constrained.

If we compare the size of the clone with position we find a significant correlation (the anterior clones are smaller, $P<0.01$) but only when the clones were induced in the young pupa (0-2 hours APF). The most anterior clones in these cases are particularly small because they are sorting from the epithelium

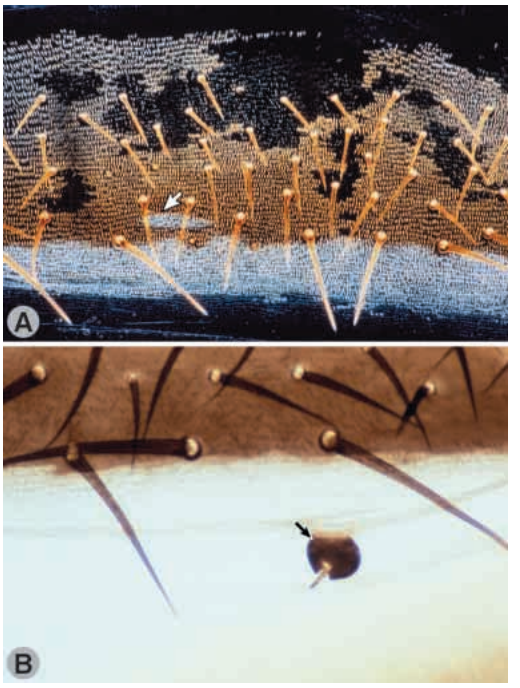

Fig. 3. Sorting out of clones. (A) If induced during late larval stages, *pwn ptc*$^{-}$ clones (arrow) only survived at the posterior part of the A compartment whereas the *sha* twins can be seen over the whole compartment. We could not pick out individual *sha* clones, they were too large and interdigitated. (B) A *pwn ptc*$^{-}$ *en*$^{-}$ in the posterior compartment. The clone (arrow) develops a5 cuticle and is partially sorted out.

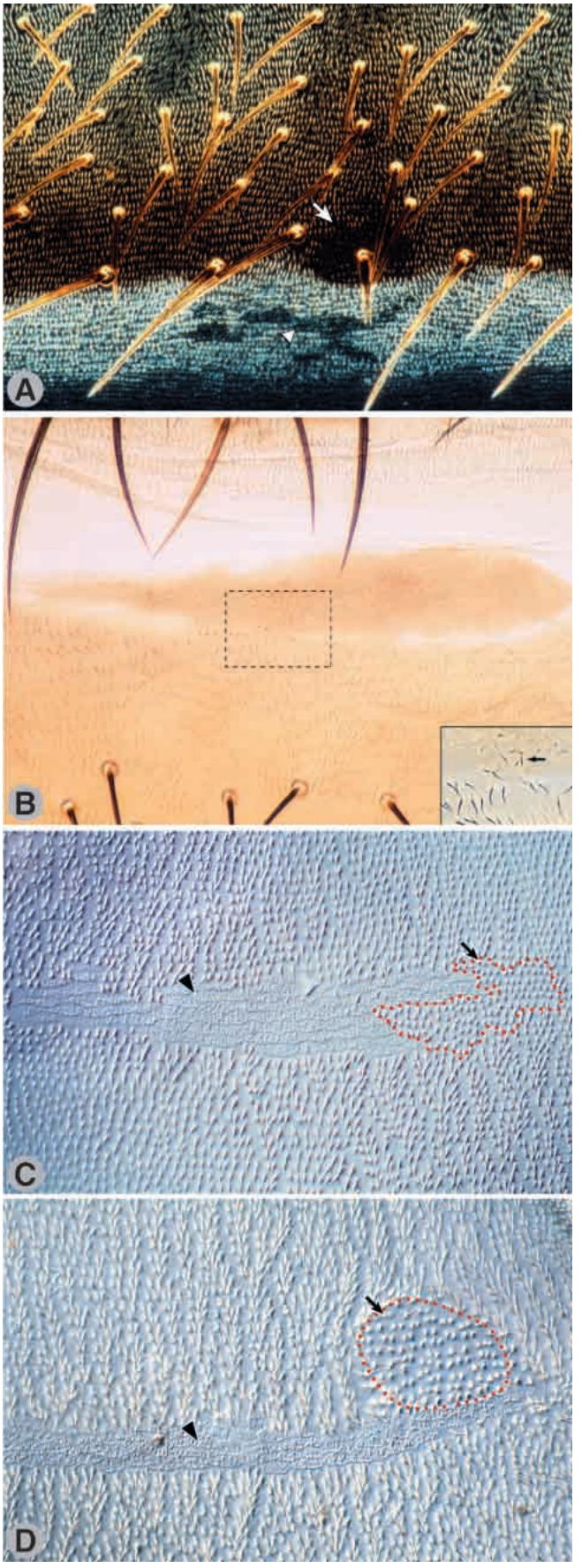

Fig. 4. Emigration from one compartment into another. (A) A *ptc*$^{-}$ *en*$^{-}$ clone, marked with *pwn*, with a *sha* twin induced in the posterior compartment at the larval stage. Note the *ptc*$^{-}$ *en*$^{-}$ cells (arrow) have fused into the A compartment in front, leaving the *sha* clone (arrowhead) behind in the P compartment. Where the *pwn* clone confronts a5 cuticle it has a wiggly boundary, but where it confronts a6 cuticle it is relatively straight. (B) A *smo*$^{-}$ *pwn en*$^{-}$ clone of P provenance in the more posterior domain of the compartment. It differentiates lightly pigmented a2 cuticle with no bristles; the hairs show reverse polarity (Lawrence et al, 1999). This clone is fusing back with the A compartment behind it. There is a narrow and interrupted band of a1 cuticle behind the clone, and here and there the fusion is witnessed by the lack of this a1 cuticle, and by the reorientation of hairs (see inset). The small arrow points to some *pwn*$^{+}$ hairs, which are not part of the clone, yet have been reoriented as a result of the proximity to mutant cells. Consider the mechanism of fusing back: the clones form a2 so the affinity should match with the cells of a2 in the compartment behind. One would think they are not in direct contact because of the 'barrier' of a1 cells. However the transformation of potential a2 to a1 cells occurs late and depends on Hh, so if there is a large A clone at the back of P, any Hh emanating from the P cells would have to traverse that clone, and could become so attenuated that it might not transform any cells behind the clone to a1 (allowing a2 cells inside and outside the clone to meet). (C) A typical *pwn* (arrow) clone (outlined with red dots) of P provenance in the pleura, with a *sha* twin (arrowhead). (D) A *smo*$^{-}$ *pwn en*$^{-}$ (arrow) clone of P provenance in the pleura, with a *sha* twin (arrowhead). We utilised the *en.FLP* transgene which generated clones only in the P compartments, mostly in the embryo. The pleura gives no outward signs of its division into A and P compartments. The *sha* twins are always long and thin and frequently fill the P compartments (which can be independently mapped with, for example, *en.lacZ*), while the *pwn* sisters are round (outlined with red dots) and partially ejected from the P compartments, in this case forwards (compare Fig. 4C).

– the correlation suggesting that the clones in the more anterior regions are more actively ejected.

4. In the posterior domain of the A compartment the twin clones are oriented: the *ptc*⁻ *en*⁻ clones are posterior to their *sha* sisters

We measured the orientation of twin clones and compared them with control *pwn*/*sha* twins. The orientation of the control twins was random ($P>0.05$); the orientation of the experimental ones was not ($P<<0.0005$). Our impression is that the more anteriorly located twins show an even stronger preferential orientation than the posteriorly located ones. Usually the twins remain in contact with each other, but sometimes there is a small gap between them.

Of 27 *ptc*⁻ *en*⁻ clones studied in the A compartment, only in one case was the centroid of the clone anterior to its *sha* twin. In this single exception, and in most similar cases from other experiments, the *sha* twin is in the a6 region, and the *pwn ptc*⁻ *en*⁻ sister, which makes a5 cuticle, is more anterior and is in, or near, a5 territory. It appears that the mutant clone is moving so as to be with cells like itself, which, in these rare cases, are anterior to the control twin.

5. In the anterior domain of the A compartment the twin clones are oriented: the *ptc*⁻ *en*⁻ clones (a1 cuticle) are anterior to their *sha* sisters

In those most anterior twin clones in which the *ptc*⁻ *en*⁻ clone forms entirely a1 cuticle in an a2 surround, we find the mutant clone to be always located adjacent but anterior to its *sha* twin (n=10) (Fig. 2C). We do not have so many cases of these types of clones: we suspect that, when the clones are induced at stages prior to 10 hours APF, the a1 clone fuses with the a1 territory and cannot be seen (the a1 cuticle is not marked by *pwn*). After about 14 hours APF, marked clones show no *ptc*⁻ phenotype.

6. Some clones span both anterior and posterior domains of the A compartment

Some single *ptc*⁻ *en*⁻ clones induced in larvae consist of an anterior part that forms a1 cuticle and a larger posterior part that forms a5 cuticle, with a trail of marked cells in between (see Fig. 5 in Struhl et al., 1997a which shows a *PKA*⁻ clone with the same phenotype). It appears that these clones have been tugged into two parts; we now think this is due to the two ends, which reside in different domains, migrating in opposite directions.

(iii) Twin clones in the posterior domain of the A compartment; the mutant clone makes a6 cuticle (*pwn ptc*⁻/*sha*)

These clones make a6 cuticle (Lawrence et al., 1999) and tend to sort out or back, leaving their *sha* partners behind (Fig. 2B). Our impression is that these clones sort out more vigorously than the *ptc*⁻ *en*⁻ ones, so that, after larval heat shocks, the few surviving clones tend to be even more restricted to the back of the A compartment. Such clones are much smaller and more circular than their *sha* twins (Fig. 3A).

(iv) Twin clones in the posterior domain of the A compartment; the mutant clone makes a3 cuticle (*pwn smo*⁻/*sha*)

Clones of this genotype were made in larvae and embryos; because of the long perdurance of *smo*⁺, the full phenotype, when *smo*⁻ cells form perfect a3 cuticle, was only seen after heat shocks made in embryos. Clones in the centre of the A compartment are completely normal (Struhl et al., 1997a).

We were most interested in clones that originated in the back part of A, and their *sha* sisters. The most posterior *smo*⁻ clones generally moved back somewhat, leaving their twins anterior to them; they came to lie in territory that straddles the normal position of the A/P border. They look ‘unhappy’ where they are, in that they assume an elliptical shape. Also, their boundaries are smooth, both where they abut the a6 or a5 territory anterior to them, and where they face the P territory behind them (Fig. 7 in Struhl et al., 1997a). We do not see wiggly boundaries where these clones meet P cells, as has been found in the wing (Rodríguez and Basler, 1997; but see Blair and Ralston, 1997). Their shape can be contrasted with their *sha* twins that do have typically irregular outlines. It seems that the affinities of the *smo*⁻ clones differ from both the A cells immediately anterior to them and from the P cells posterior to them.

Some of the *smo*⁻ clones are entirely within the A compartment, but extend from the a5 to the a3 regions; in the a3 territory their borders are wiggly. But in those parts of the clones where they contact a5 cuticle, the borders are straighter. Commonly in these cases the *sha* clone is behind its twin, extending along the most posterior part of the A compartment and colonising a6 and a5 territory. Since in control *pwn*/*sha* twins it is most usual for the twin clones at the back end of the A compartment to be side by side, our interpretation is that these mutant clones have moved forward to mingle with cells more like themselves.

Twin clones in the P compartment

(i) Twin clones in the P compartment; the mutant clone makes a5 cuticle (*pwn ptc*⁻ *en*⁻/*sha* or *pwn en*⁻/*sha*)

The P compartment contains three types of cuticle, p3-p1. Like the A compartment it is subdivided into two domains; this is shown by the behaviour of *en*⁻ clones, which transform anterior P cells to a5 and posterior P to a1. The *en*⁻ clones form a5 and a1 cuticle because Hh floods into them from the surrounding P cells (Lawrence et al., 1999). Control P clones in the P compartment are variable in shape with wiggly boundaries. But mutant *en*⁻ clones in all parts of the P compartment become elliptical or round in shape with smooth outlines, quite different from their twins (Fig. 2D).

Mutant clones induced in the larval period or earlier rarely survive in the P compartment, although they are frequent in the A. Surviving clones are always found anteriorly located, suggesting that their affinities are more compatible there. Most of these early clones fuse with the A compartment, becoming integrated into the a5 cuticle (Fig. 4A). We know these have originated in P by two criteria: first they leave their *sha* twins behind in the P compartment, and second, they form pattern characteristic of the next A compartment back (Lawrence et al., 1999).

Clones made in the pupa survive better; and particularly those in the anterior region of P. They can sometimes be found partially pinched off, only a short step from forming a separated vesicle (Fig. 3B). The position of the *sha* clone is almost invariably posterior to its *en*⁻ sister ($P<<0.0005$),

suggesting that the *en*$^-$ clone has migrated forwards (Fig. 2D). Sometimes there is a substantial gap between the twins, of up to about 5 cell diameters, with the *en*$^-$ clone even further forwards. The *en*$^-$ clone is smaller (P<0.0005), on average about half the size, and more circular (P<<0.0005) than its twin, illustrating its affinity difference from the P cells around it. Clones made in the pupa about 12 hours or more after puparium formation are small but are only partially transformed to A (presumably due to perdurance of the *en*$^+$ product). Such clones can be found all over the P compartment.

(ii) Twin clones in the P compartment; the mutant clone makes a3 cuticle (*pwn smo*$^-$ *en*$^-$/*sha*)

In this experiment the cells of the *en*$^-$ clone are also *smo*$^-$ and cannot respond to Hh. Because of the long perdurance of *smo*$^+$, the complete phenotype is only seen in clones induced in the early embryonic stages. In this case we rarely find clones in the P, we assume they have sorted out, but the small number of *smo*$^-$ *en*$^-$ clones that do survive form a3 (see Fig. 5 in Lawrence et al., 1999) or a2 cuticle. They tend to be elliptical with smooth boundaries.

However, even if induced in larvae, *smo*$^-$ *en*$^-$ clones do show mutant phenotype; they have less pigment and some of the bristles are small. We find that *smo*$^-$ *en*$^-$ clones survive better than *en*$^-$ clones, especially in the back parts of P (see Materials and Methods); indicating that a3 and posterior P cells have more in common than a5 and posterior P cells. Note that the boundaries of these a3 clones are everywhere considerably straighter than that of the *sha* twins, suggesting that there is still an affinity difference between them and every part of the P compartment. An affinity difference which we ascribe to *en*, but which could still depend on Hh, courtesy of some brief and/or residual function of *smo*$^+$.

Larval-induced clones are visible in both domains of the P compartment: in the anterior domain (about two-thirds of the P) they form a3 cuticle, with occasional larger bristles, while in the posterior domain they form a2 cuticle, without bristles. These latter clones can be very close to the anterior limit of the A compartment behind, and in some cases appear to be partially fused, or fusing with it (Fig. 4B). Such clones appear to have moved back – there can be traces of a *sha* twin anterior to the clone (remember *sha* cannot be identified in the posterior parts of the P compartment because these parts are bald).

(iii) Twin clones in the P compartment of the pleura: *pwn smo*$^-$ *en*$^-$/*sha*

This picture of now-A clones moving both forwards and backwards out of the P compartment is reinforced by making P clones in the pleura. The pleura is a lateral part of the abdominal cuticle, and consists of an elegant but featureless lawn of hairs. *pwn/sha* control twins or their sisters are elongated within the P (Fig. 4C) while the *pwn smo*$^-$ *en*$^-$ experimental clones are rounded and apparently embedded in the A compartments, either in front (Fig. 4D) or behind the P. By analogy with the tergites, it seems that the *pwn smo*$^-$ *en*$^-$ clones have formed ‘a3 or a2’ cells and been ejected from the P, either into ‘a5’ or ‘a6’ cells anteriorly or ‘a1’ cells posteriorly. The experiment appears to show that affinities of the mutant cells match neither with the P cells (the cells of the clone are A) nor with either of these neighbouring A cell types.

DISCUSSION

For a summary of the main results, please consult Fig. 1.

Cell affinities at the compartment boundary

It has long been thought that the straight and smooth boundary between compartments, for example the A/P boundary in the wing blade, is due to the cells on opposite sides having different cell affinities, meaning that each cell type (A or P) maximises contact with its own kind and minimises contact with cells of the other type (Morata and Lawrence, 1975). Recently, it has been shown that the Hh signal makes a strong contribution to this affinity difference. Hh, crossing over from P to A, gives the posteriormost cells of the A compartment affinities that helps make them distinct from the anteriormost cells of the P, thus ensuring that the interface is straight (Blair and Ralston, 1997; Rodríguez and Basler, 1997). Indeed, Rodríguez and Basler suggested that this Hh signal might be sufficient alone to define the compartment boundary. If this were true A cells that saw no Hh (lacking the Hh receptor component, Smo) would, in their affinities, be indistinguishable from P cells and might mix freely with them – their results tended to support this. However, from similar experiments, Blair and Ralston drew different conclusions: they found that *smo*$^-$ A cells frequently failed to mix normally with either P or A cells.

Here we look at affinities in the adult abdomen. The abdomen has an advantage over the wing in that, even in small clones, the types of cuticle being made can be assessed. Thus we note that *smo*$^-$ clones of A provenance can make the type of cuticle (a3) found in the middle of the A compartment. Such clones made near the back end of the A compartment come to lie between two sorts of alien cells. Behind them are P cells, and in front of them are posterior A cells (a6, a5). In the abdomen the results are unequivocal – the *smo*$^-$ clones fail to mix with either types of alien cells, forming straight boundaries with both. P clones lacking both *smo* and *en* can also form epidermal cells of the a3 type, and, at the front of the P compartment, these behave the same way as a3 cells of A provenance. By contrast *en*$^-$ clones of P provenance that form a5 cells cross over the boundary into A and mix with a5 cells there. We conclude that the A/P boundary in the abdomen (and presumably in the wing) depends on two independent factors: first there is a difference between A and P due to *en* and second there are differences within A due to the Hh signal (see Lawrence, 1997).

Gradients of cell affinity in the A compartment?

The Hh signal comes into each A compartment from two directions, and our results suggest that it acts to set up two opposing gradients of cell affinity. We have examined the behaviour of twin clones, one of which has a different identity from its neighbours and the other acts as a control. Our most detailed results concern the posterior domain within the A compartment.

(1) We find a spatial gradient of clone survival; clones of different positional identity sort out most readily when there is

a large disparity between their positional value and that of the surrounding cells. For example, *ptc*$^{-}$ *en*$^{-}$ clones sort out rapidly when they are induced anteriorly, while they survive well in the posterior part; the same type of clones when induced later survive further anteriorly, which suggests there is a continuous gradient of affinities.

(2) The wiggliness of the boundary made between the clone and its surrounding is a measure of the degree of affinity between the two types of cells. We note that, with *ptc*$^{-}$ *en*$^{-}$ clones induced at a certain stage in the pupa, the clones are more circular the more anterior they are located. This also suggests that the affinity changes continuously.

(3) We have further evidence for polarity in the epidermis, for, relative to its twin, the clone moves towards the level appropriate to its own differentiation – if the clone differentiates as a5 cuticle, then it moves towards the a5 region. This implies a vectorial arrow is present in the epithelium, for if the clone were simply uncomfortable in being surrounded by a uniform field of a3 cells, it might round up or sort out, but it would not migrate in a specific direction. We imagine this vector to be defined by a gradient of cell affinity; one would expect cells to take whatever opportunity they have to move in the direction that maximises their affinity with their neighbours (cf Ripoll et al., 1988). The adult abdominal epidermis develops as a fairly loose sheet and cells might be somewhat free to exchange neighbours, perhaps during mitosis.

Our results also indicate that the anterior domain of the A compartment (see Lawrence et al., 1999) correlates with an affinity gradient of the opposite polarity – accordingly, while the a5 or a6 clones in the a3 region move back, the a1 clones in the a2 region move forward.

Both these findings suggest that the prime agent responsible for affinity in the A compartment is Hh itself. The response to Hh is cell autonomous and we imagine the affinity depends on how much Hh is perceived – it is a scalar output from the Hh gradients.

Gradients of cell affinity in the P compartment?

In the main anterior region of the P compartment, cells lacking *en* both differentiate as A cells and migrate forward, suggesting there is a gradient of affinity in the P compartment. Also note that *en*$^{-}$ (now-A) clones in the compartment survive most readily in the anterior part of the P, presumably because their affinities are more similar to anterior than to posterior P cells. This implies some commonality between neighbouring regions of the A and P compartments, so that a5 A cells have more affinity to anterior P (p3) than they do to posterior P (p2, p1). In the posterior domain of the P compartment some cases suggest that *en*$^{-}$ clones making a1 cuticle (or *smo*$^{-}$ *en*$^{-}$ clones making a2 cuticle) move backwards. Perhaps the two domains of the P compartments, like those of the A, are each associated with affinity gradients? We do not know what agent patterns the P compartment, but most likely there will be a morphogen that acts as Hh does in the A compartment, regulating both differentiation and affinity.

Cell identity and affinity

The segmental state of a cell (such as whether it is abdominal or thoracic) depends on selector genes such as the *Bithorax Complex* (Lewis, 1978). Such genes are also responsible for giving the cells of different segments different affinities, for example when the *Ultrabithorax* gene is removed from haltere cells, they develop as wing cells and sort out from their haltere neighbours (Garcia-Bellido, 1975; Morata and Garcia-Bellido, 1976). There are thus at least three different affinity labels which act independently and additively: those dependent on the segment, on the compartment (A or P) or on position within the compartment. We guess that there may also be a label associated with the distinction between dorsal and ventral epidermis.

Why have gradients of cell affinity?

Our general model is that morphogen gradients define basic aspects of pattern: positional information is encoded in the scalar of the primary gradient (Lawrence and Struhl, 1996), information relating to size and growth in the steepness (Lawrence et al., 1996) and polarity encoded in the vector of a secondary gradient (Struhl et al., 1997a). To this we may now add the hypothesis that affinity is also encoded in the scalar, giving a graded readout, perhaps in the amount of a homophilic adhesion molecule such as a cadherin.

We think that gradients of cell affinity will prove to be basic properties of all cell sheets in vivo, where they act to ensure the integrity and stability of the sheet by keeping the cells adherent to their neighbours and reducing any tendency to roam. Without this gradient, even if all cells tended to cohere to each other, their intrinsic motility could allow them to move around by exchanging equally adhesive neighbours. Mobility like this could compromise pattern formation; it might be problematic if cells were to receive information of position from, for example, the ambient level of Hh, begin to respond to it, and then migrate to a different position too late to readjust their response.

The stripes of different types of cuticle in the A compartment are a consequence of threshold responses to a continuously varying Hh concentration. In general, once differentiation has begun in any group of cells (such as one of these stripes) they might acquire additional affinity label(s) that would reduce mixing with neighbours, thus sharpening the border line. Maybe this explains the straightness of the line between a5 and a6 cuticle, which seems straighter than one would expect if the a5 and a6 cells were mixing as much cells do elsewhere.

In the wing, the Hh gradient is responsible for pattern only close to the A/P boundary, with differentiation of cells further away in thrall to a gradient of Decapentaplegic (Dpp) (Lecuit et al., 1996; Nellen et al., 1996). There is some evidence that the gradient of affinity extends into parts of the wing outside the Hh territory: for example, clones of activated receptor for Dpp take up a circular shape (Nellen et al., 1996), showing that their cell affinities are different from the surround. Thus affinity changes may accompany positional information even when it depends on more than one morphogen.

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