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Smoothened-mediated Hedgehog signalling is required for the maintenance of the anterior-posterior lineage restriction in the developing wing of *Drosophila*

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SUMMARY

It is thought that the posterior expression of the 'selector' genes *engrailed* and *invected* control the subdivision of the growing wing imaginal disc of *Drosophila* into anterior and posterior lineage compartments. At present, the cellular mechanisms by which separate lineage compartments are maintained are not known. Most models have assumed that the presence or absence of selector gene expression autonomously drives the expression of compartment-specific adhesion or recognition molecules that inhibit intermixing between compartments. However, our present understanding of Hedgehog signalling from posterior to anterior cells raises some interesting alternative models based on a cell's response to signalling. We show here that anterior cells that lack *smoothened*, and thus the ability to receive the Hedgehog signal, no longer obey a lineage restriction in the normal position of the anterior-posterior boundary. Rather these clones extend into anatomically posterior territory, without any changes in *engrailed*/*invected* gene expression. We have also examined clones lacking both *en* and *inv*; these too show complex behaviors near the normal site of the compartment boundary, and do not always cross entirely into anatomically anterior territory. Our results suggest that compartmentalization is a complex process involving intercompartmental signalling; models based on changes in affinity or growth will be discussed.

Key words: compartment, cell lineage, selector gene, appendage patterning, *engrailed*, *invected*, *smoothened*, *hedgehog*, *cubitus interruptus*

INTRODUCTION

Appendages in *Drosophila* are derived from imaginal discs (reviewed in Cohen, 1993; Blair 1995). These epithelial sacs are set aside during embryogenesis as anlage of about 20-40 cells each; during larval life they grow to form mature discs that, prior to metamorphosis, contain tens of thousands of cells. Cell lineages are not strictly stereotyped during much of disc development. However, most discs contain one or more precisely defined lineage boundaries that cells cannot cross, subdividing the discs into lineage compartments. While much is understood about the genetic control of compartmentalization, the cellular, mechanistic bases for these strict lineage restrictions are still unknown.

It is thought that the expression of 'selector' genes controls in some manner the formation and maintenance of lineage compartments (Garcia-Bellido, 1975; Crick and Lawrence, 1975; Lawrence and Morata, 1976a; Fig. 1A). These genes are initially expressed in the precursors that give rise to one compartment, and the stable inheritance of that expression acts as a binary switch, both giving those cells a compartment-specific identity and preventing those cells from intermixing with or displacing non-expressing cells in the other compartment. For the early-arising anterior-posterior (A/P) compartment boundary in the growing wing disc, the posterior expression of the similar homeobox transcription factors encoded by *engrailed* (*en*; Kornberg et al., 1985; DiNardo et al., 1985; Brower et al., 1986; Blair, 1992) and *invected* (*inv*; Coleman et al., 1987) selects the posterior compartment identity and lineage. Loss or reduction of *en* activity in posterior cells reduces their ability to obey the A/P lineage restriction: clones lacking *en* commonly straddle the site of the normal A/P boundary (Morata and Lawrence, 1975; Lawrence and Morata, 1976b; Kornberg, 1981; Lawrence and Struhl, 1982; although see Hidalgo, 1994). The dorsally expressed transcription factor encoded by *apterous* acts as selector gene for the later-arising dorsoventral (D/V) compartment boundary: dorsal cells that have lost *apterous* cross completely into the ventral compartment (Blair et al., 1994) and form ventral-like tissues (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994).

The most widely accepted hypothesis of how selector genes control compartmentalization is that the cells throughout each compartment express, under the direct control of these genes, compartment-specific adhesion or recognition molecules (see Lawrence and Morata, 1976a,b; Kornberg, 1981; Lawrence and Struhl, 1982). Here, this will be termed the 'selector-affinity model' (Fig. 1B). In this model, cells in one compartment do not adhere to or recognize cells in the adjacent com-

partment and minimize contact with them, creating a sharp, smooth boundary between selector-expressing and non-expressing cells. Adhesion assays have provided little evidence for gross adhesive differences between cells in different compartments (Fehon et al., 1987; Fausto-Sterling and Hsieh, 1987). However, negative evidence generated using dissociated cells must be viewed with caution, and it is likely that more subtle forms of cell recognition would not be recognized by such assays. Nonetheless, it should be pointed out that no good candidate compartment-specific adhesion or recognition molecules have yet been described. Different integrin α chains do show D/V-specific expression, but this specificity arises too late in development to account for the lineage restriction (Brower et al., 1985), and removal of the shared integrin β chain does not induce cells to cross the D/V boundary (Brower and Jaffe, 1989).

Selector genes play another role during development, and that is to control signalling between adjacent compartments. Posterior cells express, under the control of *en* and *inv*, the secreted morphogen encoded by *hedgehog* (*hh*; Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992, 1995; Sanicola et al., 1995; Zecca et al., 1995). Since only anterior cells appear capable of receiving the Hedgehog signal and the signal has a limited range, this results in the formation of specialized cells just to the anterior of the A/P boundary. Anterior boundary cells react by expressing the growth factor encoded by *decapentaplegic* (*dpp*) and heightened levels of the transmembrane protein encoded by *patched* (*ptc*), and by stabilizing the transcription factor encoded by *cubitus interruptus* (*ci*) (Basler and Struhl, 1994; Tabata and Kornberg, 1994; Slusarski et al., 1995; Schwartz et al., 1995; Zecca et al., 1995; Guillen et al., 1995; de Celis and Ruiz-Gomez, 1995). Late in larval development these cells also express *en*; this paradoxical expression of *en* in the 'wrong' compartment apparently arises too late in devel-opment to affect the A/P lineage restriction (Blair, 1992). Experi-ments using ectopic Hh signalling or reductions of Fused activity suggest that Hh signalling is required for expression of *en* just to the anterior of the A/P boundary (Tabata et al., 1995; Guillen et al., 1995; de Celis and Ruiz-Gomez, 1995; Sanchez-Herrero et al., 1996; Gomez-Skarmeta and Modolell, 1996; Mullor et al., 1997). This requirement will be demonstrated directly below.

Most writers have assumed that intercompartmental signalling and compartmentalization are separate processes. The selector-affinity model hypothesizes that compartment-specific adhesion or recognition is under the direct, cell autonomous control of the selector genes. The model therefore predicts that alterations in intercompartmental signalling should not affect the formation or maintenance of the lineage restriction, provided that such changes in signalling do not affect selector gene expression. However, the existence of intercompartmental signalling provides some intriguing alternatives to the selector-adhesion model. In one simple alternative model (Fig. 1C), the critical difference in adhesion or affinity is not between all *en*/*inv* expressing and all non-expressing cells, but rather between cells that have received or not received the Hh signal. Cells that have received the signal would express specific adhesion or recognition molecules, and thus sort out from cells that have not received the signal. Since only anterior cells can receive the Hh signal, the affinities of posterior cells would differ from those of anterior cells at the boundary, and this would prevent intermixing. Intermixing would be permitted in the anterior because cells could either gain or lose their Hh-dependent state of affinity, depending upon their proximity to the posterior Hh signal.

According to this 'signalling-affinity' model, the ability of posterior *en*⁻ cells to cross the A/P boundary would be due, not to the autonomous loss of some adhesive or recognition molecule, but rather because posterior *en*⁻ cells are capable of receiving the Hh signal, as has been demonstrated (Sanicola et al., 1995; Zecca et al., 1995; Tabata et al., 1995). Since such

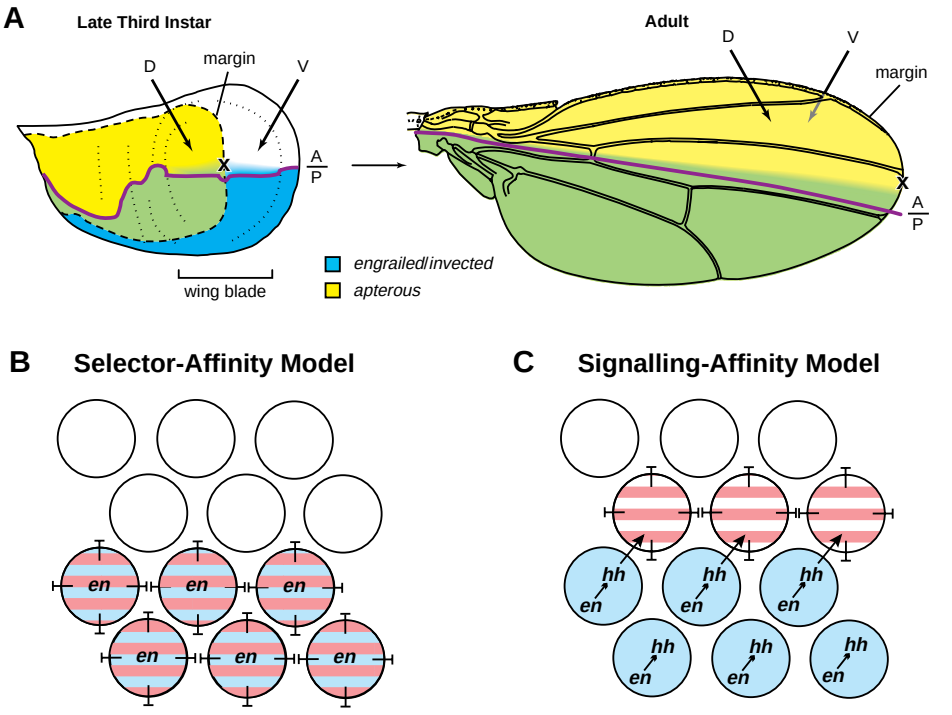

Fig. 1. (A) Lineage compartments and domains of gene expression in a late third instar wing disc and the adult wing. X marks the prospective distal tip of the wing; after late third instar the dorsal and ventral surfaces of the wing blade fold over one another to make the bi-layered adult wing. (B) The selector-affinity model of compartmentalization. *en* (and *inv*) expression in posterior cells (blue) drives the expression of compartment-specific adhesion or recognition molecules (red stripes). (C) The signalling-affinity model of compartmentalization. *en* (and *inv*) expression in posterior cells (blue) drives the expression of *hh*, which signals to anterior cells adjacent to the A/P boundary. In response, anterior cells express boundary-specific adhesion or recognition molecules (red stripes).

cells would be surrounded by *hh*-expressing cells, they would receive the signal and thus resemble in terms of affinity cells on the anterior side of the A/P boundary. The model can also explain the behavior of anterior clones distant from the A/P boundary that lack *ptc* or protein kinase A (PKA). Such clones, which autonomously mimic reception of the Hh signal, form smooth boundaries with adjacent tissues, much like those seen at compartment boundaries (Phillips et al., 1990; Capdevila et al., 1994; Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Chen and Struhl, 1996).

To test the involvement of Hh signalling in the maintenance of the A/P lineage restriction, we have interfered with the ability of cells to receive the Hh signal by removing the gene *smoothened* (*smo*). Because *smo* encodes a transmembrane protein with similarities to G-protein-coupled receptors, it was originally suggested that Smo is the Hh receptor (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Recent evidence suggests that the transmembrane protein encoded by *patched* (*ptc*; Hooper and Scott, 1989; Nankano et al., 1989) is responsible for binding Hh and that Ptc and Smo act together to transduce the signal (Stone et al., 1996; Marigo et al., 1996; Chen and Struhl, 1996). Cells lacking *smo*, however, no longer respond to Hh (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Chen and Struhl, 1996). Segment polarity defects observed in *smo*$^-$ embryos resemble those observed after loss of *hh* (although these are also similar to those expected from loss of 'autocrine' Wingless signalling in the embryo; Alcedo et al., 1996; van den Heuvel and Ingham, 1996). More tellingly, clones of cells that lie just to the anterior of the A/P boundary in the wing disc fail to express the Hedgehog target *dpp*, and anti-Ptc- and anti-Ci-staining levels are reduced to those observed in the far anterior of the compartment (van den Heuvel and Ingham, 1996; Chen and Struhl, 1996; see below).

Our results will show that the predictions of the selector-affinity model are not met, as anterior *smo*$^-$ clones show a strong tendency to extend into anatomically posterior territory, either crossing or displacing the A/P lineage boundary. However, some details of the signalling-affinity model are also not met by the data. We have also examined clones lacking both *en* and *inv*; these too show complex behaviors near the

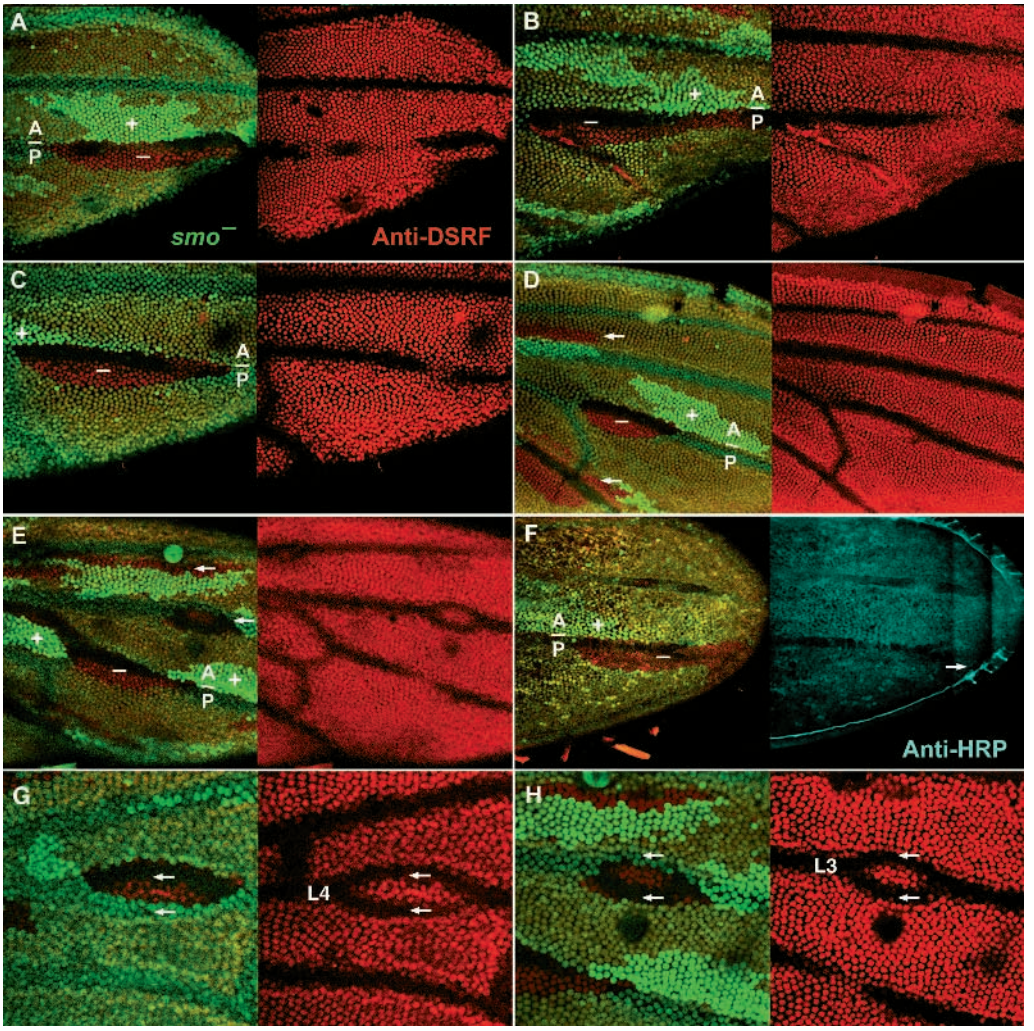

Fig. 2. Pupal wings (24-36 hours after pupariation) containing *smo*$^-$ clones, marked by the absence (–) of the πM marker (green). πM/πM twin spots (+) are visible as regions of heightened anti-Myc staining. Veins are marked by the absence of anti-DSRF staining (red). (A-D) Examples of *smo*$^-$ clones of unambiguously anterior origin, adjacent to anterior πM/πM twin spots, which have crossed or displaced the A/P boundary. Note the abnormally smooth boundaries between *smo*$^-$ and adjacent cells. In most cases there is sporadic disruption of L4-like tissue within the clone, as not all cells in this region lack anti-DSRF staining. (D) Note that the PCV avoids the *smo*$^-$ clone. A clone anterior to the third longitudinal vein (arrow) does not induce obvious abnormalities. (E) Large clone of anterior origin that straddles the normal position of the A/P boundary, lying anterior in the proximal wing and posterior in the distal wing. The ectopic venation between L3 and L4 is typical of such clones. Arrows indicate clones further anterior; the one near L3 induces ectopic venation within and around the clone (detail in H), while the one near L2 does not induce obvious abnormalities. (F) Clone of anterior origin lying largely within anatomically posterior territory. Where it is contact with the margin, anterior-like neurons are formed (arrow), as seen using anti-HRP (blue). (G) Small clone of anterior origin lying in an ambiguous position along L4. The clone induces branching in the vein (arrows), with one branch in the anterior of the clone and a second branch just to the posterior of the clone. (H) Detail of anterior clone in E, lying along L3. The clone induces branching in the vein (arrows), with one branch in the posterior of the clone and a second branch just to the anterior of the clone.

A/P boundary. Our results suggest that compartmentalization is a more complex process than can be explained by either of the simple models outlined above; alternatives based on changes in affinity and growth will both be discussed.

MATERIALS AND METHODS

Clones were generated using the FRT-FLP method (Xu and Rubin, 1993). en^E is a deletion removing all the *en* and most of the *inv* coding sequences (Tabata et al., 1995). FRT^{42B} en^E/*CyO* males were crossed to *y* w *hsFLP1*; FRT^{42B} πM females (both kindly provided by M. Singer). smo^{IIG25} is an extreme hypomorphic allele (van den Heuvel and Ingham, 1996). smo^{IIG25} FRT^{40}/*TM6,Tb* males (from a stock generated in our laboratory) were crossed to *y hsFLP1*; FRT^{40} πM females. *hh-LacZ* (hh^{R40}; Lee et al., 1992) was crossed to the *y hsFLP1*; FRT^{40} πM stock to follow *hh* expression. Unless otherwise noted, resultant larvae were given a 45-90 minute heat shock (37°C) 72 hours before being picked as wandering third instar larvae (reared at 25°C, approximately equivalent to heat shock at 48 hours after egg laying, AEL). Larvae were heat shocked again for 1.5 hours to induce πM expression; discs were dissected in *Drosophila* Ringers and fixed 2 hours in formaldehyde-Pipes-EGTA-NP40 (Brower, 1986). To obtain pupal wings, wandering third instar larvae were reared for 2 days at 20°C (equivalent to 24-36 hours after pupariation at 25°C), heat shocked for 3 hours to induce πM expression, partially dissected from the pupal case in Ringers and fixed overnight; the wings were then completely dissected from the pupal cuticle. All subsequent incubations were carried out at 4°C in PBS containing 0.3% Triton X-100 (Sigma).

In our hands, the 4D9 mouse anti-En/Inv gives high background staining in certain wild-type stocks, such as Canton-S. For this reason, we used the 4F11 mouse anti-En/Inv (Patel et al., 1989; kindly provided by N. Patel; 1/10 dilution), which gave little or no background staining. Either mouse (1/5 dilution) or rabbit anti-Myc (1/1000 dilution) was used to label clones; rabbit anti-c-Myc (Santa Cruz Biotechnology) was preadsorbed at a 1/10 dilution over fixed 0-3 hour AEL embryos (approximately 3 volumes solution to 1 volume embryos) to reduce background staining. Simultaneous staining with rat anti-Ci (Slusarski et al., 1995; kindly provided by R. Holmgren) often disrupted anti-Myc staining; such discs were therefore first stained with anti-Myc without anti-Ci, briefly postfixed and then stained with anti-Ci (1/10 dilution). Rat anti-DSRF (Affolter et al., 1994; kindly provided by M. Affolter) was used at a dilution of 1/1500 and rabbit anti-HRP (Jackson) at 1/1000.

Incubation in fluorescent secondary antibodies (RITC, FITC, Cy5) was for 3 hours total. We used 1/200 dilutions of preadsorbed, low cross reactivity fluorescent anti-IgGs (Jackson), or 1/800 dilutions of non-adsorbed fluorescent anti-IgGs (US Biochemicals). For the most critical staining, we used 1.5 hours in 1/200 biotin anti-IgG (Vector) followed by 1.5 hours in 1/1600 RITC-streptavidin (Vector). Wings were mounted in 80% glycerol-PBS containing 4% propyl gallate, and viewed using a Biorad confocal microscope. Single images were merged using Adobe Photoshop.

RESULTS

Twin-spot analysis

To test the role of genes in controlling the lineage restriction, it was necessary to determine the compartmental origin of mutant cells independent of their final position in the developing wing. This was accomplished using twin-spot analysis. Mitotic recombination in heterozygotic flies generates two homozygotic daughter cells. When marked using a ubiquitously expressed, non-endogenous epitope, the clones generated by these cells can be recognized by the absence (unmarked/unmarked) or heightened (marked/marked) expression of that marker in a heterozygotic (marked/unmarked) background. Normally, the two clones lie adjacent to each other and, because the A/P boundary in the developing wing disc anlage arises during embryogenesis, associated wild-type twin spots induced during larval life always lie on the same side of the A/P boundary. In the analysis that follows, mutant clones will only be discussed if they were unambiguously adjacent to a +/+ twin spot in the posterior or anterior compartments and thus could be assigned a compartmental origin. Clones adjacent to both anterior and posterior twin spots will not be discussed.

smo$^-$ cells of anterior origin cross or displace the A/P lineage boundary

In order to more easily determine the location of the A/P boundary, *smo*$^-$ clones were initially examined in pupal wings (24-36 hours after pupariation). Anti-DSRF (Blistered), which labels intervein regions (Affolter et al., 1994; Montagne et al., 1996), was used to determine the pattern of venation, which at this stage resembles that observed in adults.

No role for Hh signalling has been previously observed in the posterior compartment of the wing. As expected, posterior *smo*$^-$ clones of unambiguously posterior origin showed no obvious defects in venation or wing morphology (Fig. 2D), even when immediately adjacent to the A/P boundary (not shown). In the anterior compartment, Hh-dependent responses are known to extend as far anterior as the third longitudinal vein (L3, the anterior limit of *dpp-LacZ* and heightened anti-Ci staining). As expected, defects, such as loss of veins or ectopic venation, were observed in *smo*$^-$ clones within this region (Fig. 2E,H). Clones lying on or between L3 and L4 commonly induced ectopic venation both within and occasionally adjacent to the clone and had abnormally rounded shapes, as might be expected from local differences in adhesion or affinity. Small clones on L3 in some cases split the vein into anterior and posterior branches. The posterior branch always lay within the clone near its posterior boundary, while the anterior branch usually lay largely or wholly anterior to the clone (Fig. 2H). Few clones anterior to L3 had obvious defects in morphology or venation (Fig. 2D,E).

Interestingly, clones of unambiguously anterior origin, if located at the A/P boundary, only rarely defined the site of the normal A/P boundary from the anterior side (1/37 clones), either crossing the A/P or displacing it posteriorly (see Discussion). The majority of clones lay mostly or wholly within anatomically posterior territory, appearing to define from the posterior a smooth lineage restriction at the approximate site of the normal A/P (22/37 clones; Fig. 2A-D,F). In about half of these clones, the anterior boundary appeared to push slightly into anatomically anterior territory, but this was not always true, and in even these cases most of the clone clearly lay posterior to the site of the normal A/P boundary as defined by the twin spot. In a few cases, large clones straddled between anterior and posterior territories; the anterior portion in these lay proximally in the wing, while the posterior portion lay further distally and there defined from the posterior a lineage restriction at the approximate site of the normal A/P (4/37; Fig.

2E). In a few cases, especially with smaller clones, the position of the clone was more ambiguous, and neither the anterior or posterior boundary of the clone clearly defined the site of the normal A/P (10/37; Fig. 2G).

Normally, the fourth longitudinal vein (L4) is located just posterior to the A/P boundary. All clones that completely crossed or displaced the A/P boundary contained vein tissue in the approximate position of the normal L4, contiguous with the normal L4 outside the clone. While some clones induced abnormalities and distortions in anterior and posterior venation, the majority had only slight defects, including sporadic loss of the L4-like venation, despite being formed of originally anterior tissues. In the class of smaller, ambiguously positioned clones, L4 often was split into two branches. The anterior branch always lay within the clone near its anterior boundary, while the posterior branch usually lay largely or wholly posterior to the clone (Fig. 2G). Similar branching was not consistently induced around larger clones (Fig. 2A-C,E,F), although in a few cases vestigial posterior branches were observed (Fig. 2D).

smo⁻ cells of anterior origin retain anterior-like features in posterior territory

Although anterior *smo*⁻ cells can extend into anatomically posterior territory, they retain anterior-like features. Interestingly, in no cases did clones of anterior origin form posterior cross vein (PCV); rather, the PCV appeared to avoid clone tissue (Fig. 2D).

Normally, neurons are only found in the anterior compartment: neurons of the campaniform sensilla are found only on L3 and L1, and the posterior margin bristles are aneural (Murray et al., 1984). When neurons were labeled in 24-36 hour AP wings with anti-HRP, neurons were occasionally found on the *smo*⁻ L4-like veins and, when *smo*⁻ clones of anterior origin were found along the margin, they almost always contained neurons (Fig. 2F). Thus, while lying in the normal position of L4, the vein had on occasion some L3-like characteristics.

Adult wings containing unmarked *smo*⁻ clones were also examined. The phenotypes observed were all consistent with the defects observed in pupal wings, including the defects in venation, the formation of campaniform sensilla on 'L4' and the transformation of margin bristle types (not shown).

One explanation for the behavior of anterior *smo*⁻ clones is that clones have changed their selector gene expression. It is known that disc cells can alter their compartmental fates during regeneration (Abbott et al., 1981), presumably by altering selector gene expression, and it is possible that the loss of *smo* could be inducing similar effects. However, the anterior-like features retained by the clones argue against this, and further examination of compartment-specific gene expression in late third instar discs shows that *smo*⁻ clones of anterior origin retain their anterior identity in the posterior. At this stage, posterior cells do not stain with anti-Ci, but do stain with anti-En/Inv and express *hh-LacZ*. In the absence of *smo*, anterior cells at this stage do not stain with anti-En/Inv or express *hh-LacZ*, and have low but detectable levels of anti-Ci staining; this was true even of anterior clones that had extended into anatomically posterior territory (Fig. 3). This agrees with the results of Chen and Struhl (1996).

smo⁻ cells of anterior origin do not associate normally with posterior cells

The results above show that Hh signalling plays a strong role in maintaining or localizing the normal A/P lineage restriction without altering selector gene expression or anterior-posterior identity. This favors models like the signalling-affinity model (Fig. 1C). In one respect, however, the predictions of the signalling-affinity model were not met. Normally, wild-type clone boundaries are ragged and cells inside and outside the clone interdigitate, as long as the boundary does not coincide with either the A/P or D/V lineage restriction (see +/+ twin spots in Figs 2-5). However, anterior *smo*⁻ clones did not interdigitate normally with posterior cells. *smo*⁻ clones of unambiguously anterior origin had abnormally smooth boundaries with neighboring posterior cells (Figs 2,3). This was apparent both in late third instar discs and pupal wings. Thus, although anterior *smo*⁻ cells normally lie within anatomically posterior territory, they did not associate normally with either anterior or posterior cells.

Comparison with clones lacking *en* and *inv*

Previous studies show that the loss of *en* does not cause a perfect loss of the A/P lineage restriction. If *en*⁻ cells transformed into anterior-like cells, then they should cross entirely into the anterior compartment and obey the lineage restriction from the anterior side. While parts of some *en*⁻ clones may act this way, other parts straddle the normal site of the A/P boundary (Kornberg, 1981; Lawrence and Struhl, 1982). One explanation for this behavior is that the transformation is partial, due to the redundant requirement for the related homeobox gene *invected* (*inv*). Cells lacking *en* alone only partially transform from posterior to anterior identities in terms of compartment-specific tissue patterns and gene expression (reviewed in Blair, 1995). Removal of both *en* and *inv* improves the transformation, indicating that the two genes play redundant roles in specifying posterior identity (Hidalgo, 1994; Sanicola et al., 1995; Zecca et al., 1995; Tabata et al., 1995; Simmonds et al., 1995; Gustavson et al., 1996).

However, Hidalgo (1994) showed that some posterior *en*⁻ *inv*⁻ clones obeyed the A/P lineage restriction, suggesting that *en* and *inv* cannot entirely account for A/P compartmentalization. One difficulty with that study was that there was no independent marker of the compartmental origin of the clones. Thus, clones that were originally induced in the posterior compartment and then crossed perfectly into the anterior would have been identified as anterior rather than posterior clones. We have repeated these experiments using twin-spot analysis to identify the compartmental origin of the clones.

en⁻ *inv*⁻ double mutant clones induced at 48 or 72 hours before pupariation, and examined in pupal wings (24-36 hours after pupariation). *en*⁻ *inv*⁻ clones of posterior origin showed a wide variety of phenotypes. As expected from previous studies, clones distant from the A/P boundary induced outgrowths and duplications both in and adjacent to the clones (Fig. 4B,E), presumably due to the ectopic *dpp* expression induced in such clones (Sanicola et al., 1995; Tabata et al., 1995; Zecca et al., 1995). Clones at the A/P boundary also showed a range of phenotypes. Some clones of posterior origin crossed into or displaced the A/P boundary entirely and formed from the anterior a lineage restriction at the site of the normal A/P boundary (25/50 clones; Fig. 4A,B). Many posterior clones, however, straddled the normal site of the A/P restriction (22/50, Fig. 4C,D). In some cases such clones were associated with outgrowths and extra or disrupted venation at the site of the normal A/P boundary, making it difficult to determine if such

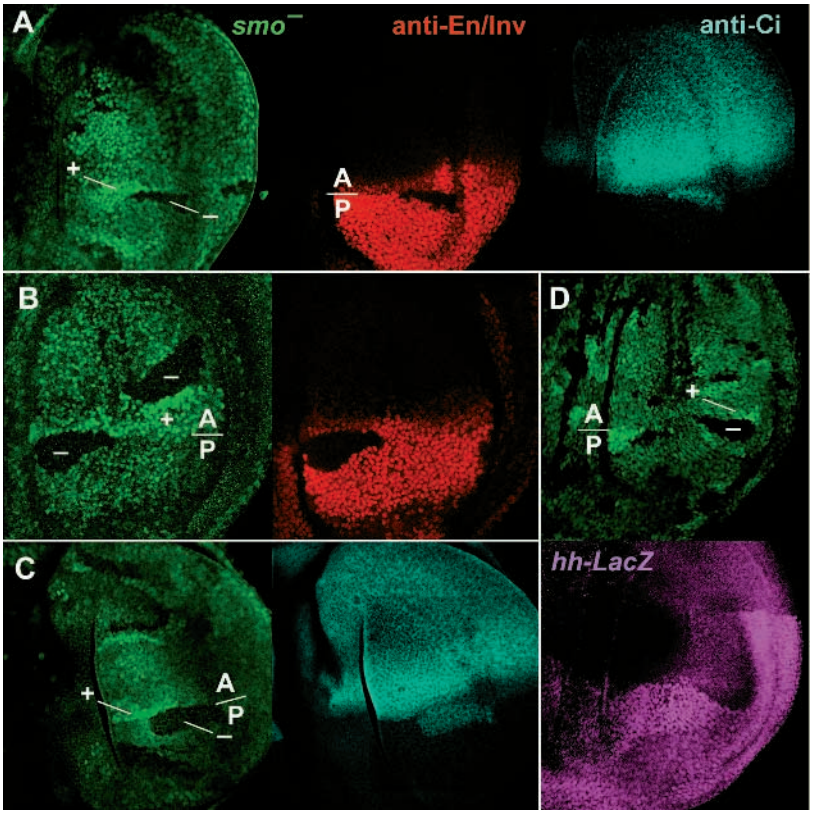

Fig. 3. Late third instar wing discs containing *smo*⁻ clones, marked by the absence (–) of the πM marker (green). πM/πM twin spots (+) are visible as regions of heightened anti-Myc staining. The A/P boundary was located by the boundary of anti-Ci or *hh-LacZ* staining, by the shapes of + clones, or by the slightly diminished anti-En/Inv staining (Blair, 1992). Note, however, that at this stage there is substantial anterior anti-En/Inv staining, and that anti-Ci staining is slightly lowered where it overlaps anti-En/Inv staining. (A) A clone of anterior origin lacks anti-En/Inv staining (red) but stains at low levels with anti-Ci (blue). (B) Clones of anterior origin, one of which lies largely in anatomically posterior territory (left). Both lack anti-En/Inv staining (red). (C) Clone of anterior origin which lies largely within anatomically posterior territory and stains at low levels with anti-Ci (blue). (D) Clone of anterior origin which lies within anatomically posterior territory, and lacks expression of *hh-LacZ* (anti-β-gal, purple).

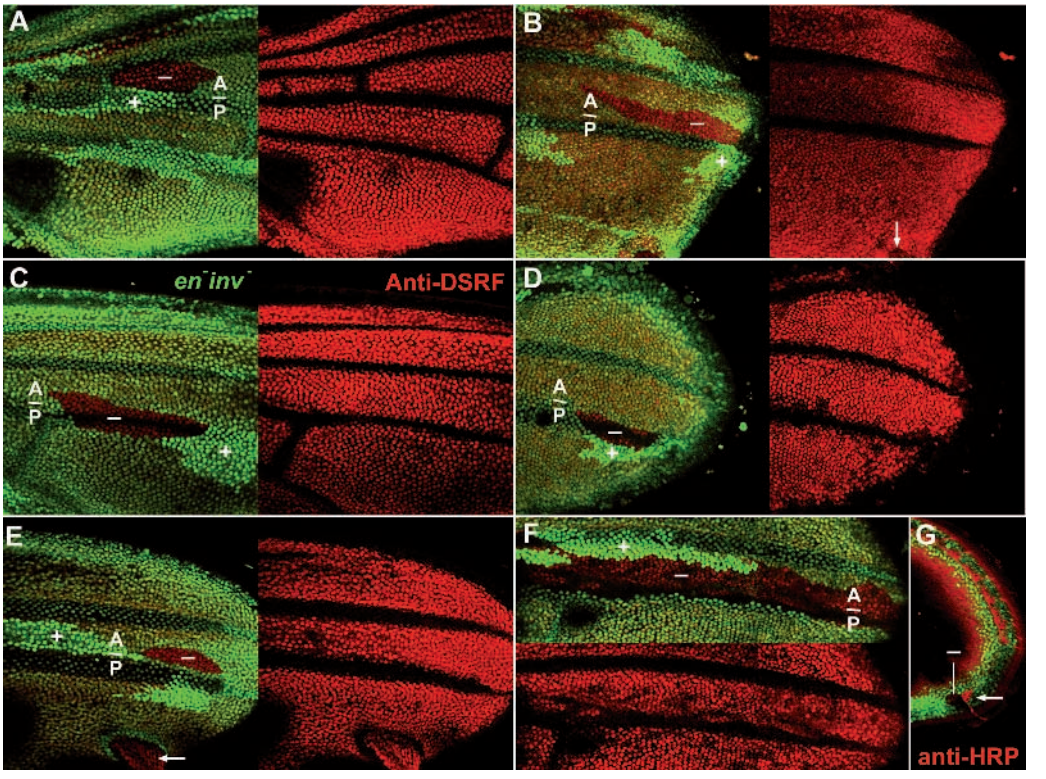

Fig. 4. Pupal wings (24-36 hours after pupariation) containing *en*⁻ *inv*⁻ clones, marked by the absence (–) of the πM marker (green). πM/πM twin spots (+) are visible as regions of heightened anti-Myc staining. In all except (G), veins are marked by the absence of anti-DSRF staining (red). (A,B) Examples of clones of posterior origin which lie entirely within anatomically anterior territory, defining from the anterior a lineage boundary in the normal position of the A/P. (B) Note abnormal growth of posterior compartment, apparently induced by posterior clone (arrow). (C) A clone of posterior origin which straddles the normal site of the A/P boundary. (D) A clone of posterior origin which defines from the posterior a lineage boundary in the normal position of the A/P. (E) A clone of anterior origin, just anterior to the A/P boundary, with an abnormally smooth clone boundary. A posterior clone (arrow) has induced a small outgrowth. (F) A clone of apparently anterior origin (note anterior twin spot) which appears to have extended into anatomically posterior territory. Note that the clone has a ragged posterior boundary and includes L4-like tissue. (G) Posterior clone on the wing margin, containing an anterior-like neuron (arrow, anti-HRP, red).

clones truly crossed or displaced the boundary (10/50; not shown, see Hidalgo 1994). This was especially common in proximal clones. Other clones, however, straddled the site of the boundary with little or no disruption to the pattern of venation (12/50; Fig. 4C). Finally, in a few rare cases, clones appeared to obey the A/P lineage restriction from the posterior side (3/50; Fig. 4D).

en⁻ *inv*⁻ clones do not associate normally with anterior cells

As with *smo*⁻ clones, posterior *en*⁻ *inv*⁻ clones that had crossed or displaced the A/P boundary rarely interdigitated with neighboring anterior cells. Rather, most clones had abnormally rounded boundaries, just as they do within the posterior compartment (Fig. 4A-C). This, and the ability of some posterior clones to obey the A/P restriction (Hidalgo, 1994; Fig. 4D), suggests that any transformation to anterior-like fates is incomplete.

Anterior *en/inv* expression and *smo*

Between mid and late third instar, *en*/*inv* expression appears in the prospective wing blade just anterior to the A/P boundary, extending at the distal tip from the A/P almost up to L3 (Blair, 1992). One function of this expression is to suppress on the margin the formation of anterior-like sensory bristles, producing instead aneural posterior-like margin bristles between L3 and L4 (Hidalgo, 1994). As expected, *en*⁻ *inv*⁻ clones along the margin between L3 and L4, whether of anterior or posterior origin, caused these normally non-sensory bristles to form neurons like those observed further anterior (not shown). Similar transformations were observed in clones along the posterior margin (Fig. 4G).

It has also been suggested that the loss of anterior *en* and *inv* expression can reduce the distance between L3 and L4 (Hidalgo, 1994). While we did not observe this, some *en*⁻ *inv*⁻ clones of anterior origin between L3 and L4 did have abnormally rounded shapes and smooth boundaries (Fig. 4E), suggestive of some additional function for *en/inv* expression in this region. This was not, however, observed in all clones and most appeared normal (see the ragged anterior boundary of the clone in Fig. 4F). In two cases, a clone adjacent to an anterior twin spot extended into and posterior to L4, in one case disrupting the L4 pattern (Fig. 4F), apparently violating the A/P restriction from the anterior side. While it is possible that these cases were fusions of anterior and posterior clones, no posterior twin-spots were found.

Interestingly, margin *smo*⁻ clones that remained in the anterior region between L3 and L4 also formed neurons (not shown). It has been suggested elsewhere that the anterior expression of *en*/*inv* is Hh dependent, like other types of boundary-specific gene expression (see Introduction). When small *smo*⁻ clones were observed at late third instar, clones just anterior to the A/P boundary not only autonomously reduced anti-Ci staining (Fig. 5A; see Chen and Struhl, 1996), but also lost anti-En/Inv staining (Fig. 5B). Thus, the formation of neurons by margin *smo*⁻ clones between L3 and L4 is almost certainly due to the loss of late-arising *en*/*inv* expression. Posterior *smo*⁻ clones did not affect posterior anti-En/Inv staining.

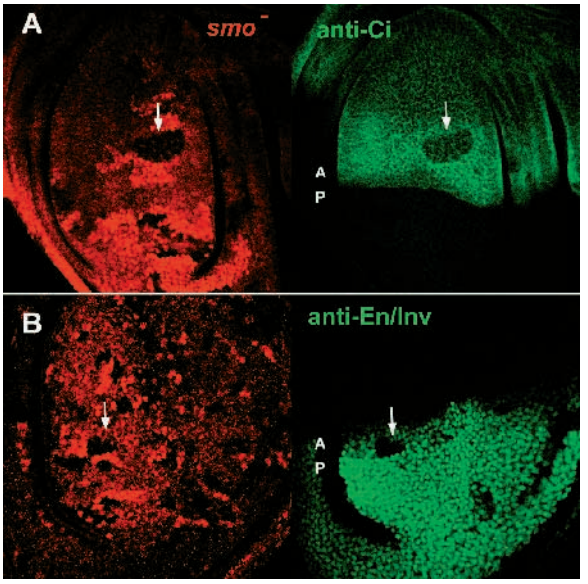

Fig. 5. Late third instar wing discs containing small *smo*⁻ clones, marked by the absence of the πM marker (red). (A) Anti-Ci staining (green), normally at higher levels in anterior cells near the A/P compartment boundary, reduced in a *smo*⁻ clone (arrow) to levels typical of anterior cells distant from the A/P. (B) Anti-En/Inv staining (green) in the anterior compartment, as marked by the line of slightly diminished staining anterior to the A/P (Blair, 1992). This expression is eliminated within a *smo*⁻ clone (arrow). Posterior anti-En/Inv staining is not altered within clones.

DISCUSSION

The results presented above show that, contrary to the selector-affinity model, Hh signalling plays a strong role in keeping

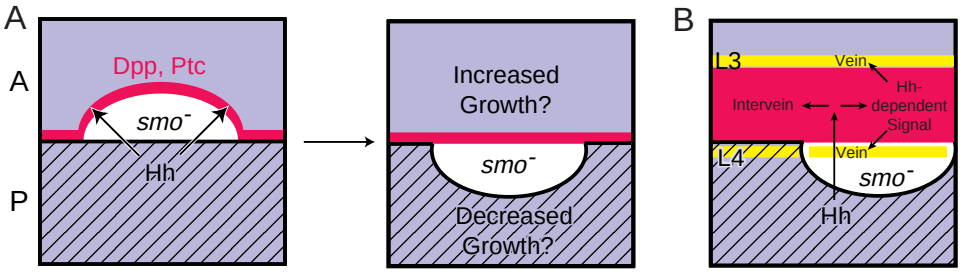

Fig. 6. (A) A growth-based model for the behavior of anterior *smo*⁻ clones. (Left) Hh induces a new region of boundary-like gene expression (red) to the anterior of the *smo*⁻ clone (Chen and Struhl, 1996). (Right) Cells surrounded by such an anterior boundary regulate the size and shape of the compartment, pushing the *smo*⁻ cells into the posterior of the wing. See text for discussion. (B) A model of vein formation. Hh is produced in the posterior and is received by cells to the anterior of the boundary. These cells (red) respond by autonomously suppressing vein formation, forming instead intervein tissue, and by producing a second, Hh-dependent signal. Cells incapable of receiving (posterior, *smo*⁻) or out of range (anterior) of the Hh signal respond to the second Hh-dependent signal by forming vein tissue (yellow).

cells within anatomically anterior territory. Anterior cells that cannot receive the Hh signal can cross or displace the A/P lineage boundary. This ability to extend across the normal site of the boundary is not caused by changes in selector gene expression. *smo*$^-$ cells of anterior origin that lie in anatomically posterior territory do not express detectable levels of the posterior-specific selector genes *en* and *inv*. They also express the low but detectable levels of *ci* typical of anterior cells distant from the A/P boundary.

This result has implications not only for imaginal disc development, but raises the possibility that similar event might underlie some aspects of the segment polarity phenotypes observed after the loss of Hh signalling in *Drosophila* embryos. As in the disc, it has been assumed in most embryonic studies that loss of signalling across the parasegmental boundary affects epithelial gene expression and cell fate without altering the positions of adjacent cells. If signalling does play a role in the embryo similar to that observed here in the wing disc, then some degree of intermixing between anterior and posterior cells would be expected after the removal of Hh signalling components. Moreover, because of the mutual dependence observed between Hh and Wingless signalling in the embryo, disruptions to either pathway might induce the loss or alteration of parasegmental boundaries.

Crossing or displacement?

There are two different ways of interpreting this data. First, *smo*$^-$ clones of anterior origin may have changed their affinities and actively 'crossed' into the posterior compartment, much as posterior *en*$^-$ clones are thought to 'cross' into the anterior (Fig. 1B). However, it is also possible that the normal A/P lineage restriction is 'displaced' posteriorly by changes in growth in the surrounding tissues, since smooth lineage restrictions are formed both to the anterior and posterior of such clones. In this view, the posterior clone boundary, although lying in the posterior of the wing, would be the original A/P lineage restriction (see Fig. 6A). We will discuss models based on both these ideas, but first we wish to make two points. First, the anterior boundary of the *smo*$^-$ clones in question commonly formed a smooth lineage restriction in the approximate position of the normal A/P boundary. The preference of *smo*$^-$ clones for associating with the posterior of the wing was marked, even without reference to such landmarks as venation. This would not be expected of a foreign group of cells 'trapped' between compartments; our models must account for the posterior movement of these cells. Second, these clones lay in posterior territory without inducing obvious distortions or gross pattern abnormalities in the surrounding tissues, and could even form vein tissue in the position of the normally posterior L4. While previous work has demonstrated that large *smo*$^-$ clones, generated near the A/P very early in disc development, can duplicate anterior structures (Chen and Struhl, 1996), we did not observe this in our smaller clones.

Affinity-based models for compartmentalization

Anterior cells lacking *smo* move preferentially into the posterior territory of the wing. In the majority of cases, clones of anterior origin found near the A/P lay wholly or mostly within anatomically posterior territory; only in a few cases did such clones straddle the normal site of the A/P boundary. This is the expected outcome of the signalling-affinity model outlined in the Introduction (Fig. 1C), as cells unable to receive the Hh signal should now resemble posterior cells in terms of affinity. While as yet there are no good candidate molecules that would drive this Hh-dependent affinity, there exist several uncharacterized enhancer trap lines that show expression on the anterior side of the A/P boundary for some or all of its length, as would be expected of Hh-driven adhesion or signalling molecules (e.g. Brook et al., 1993; Tabata and Kornberg, 1994).

However, the association of anterior *smo*$^-$ cells with posterior cells was not perfect, as an abnormally smooth boundary was formed between posterior cells and the *smo*$^-$ cells of anterior origin. This does not agree with the pure form of the signalling-affinity model, which would predict that all cells that have not received the Hh signal should have similar affinities and thus interdigitate. One way to reconcile these results is to combine both the signalling-affinity and selector-affinity models. That is, there may be a role both for Hh-driven affinities and for direct, selector-driven affinities, in keeping the anterior and posterior cell populations separate.

It may also be that the signalling-affinity model as formulated above is overly simplistic, as it assumes that signalling only goes from posterior to anterior. We know that the Dpp secreted on the anterior side of the compartment boundary can signal back to posterior cells. Since anterior and posterior cells differ in their response to Dpp, it is entirely possible that cells both immediately anterior and posterior to the boundary are differentially specified in terms of affinity. If this were true, *smo*$^-$ clones of anterior origin would never totally resemble cells on the posterior side of the normal A/P, accounting for the smooth boundary. Interestingly, Hidalgo (1994) noted apparent distortions in the A/P lineage boundary in *dpp* hypomorphs.

Affinity and the behavior of *en*$^-$ *inv*$^-$ clones

By either of the simple affinity-based models outlined in Fig. 1, posterior cells lacking *en* and *inv* should take on an anterior-like affinity. Either they lack *en/inv* driven adhesion or recognition molecules, or they are now able to receive the Hh signal and so take on affinities like those on the anterior side of the A/P boundary. However, the work of Hidalgo (1994) and our own study show that the transformation induced by the lack of *en* and *inv* is imperfect. While some clones completely cross from posterior to anterior territory, other clones only straddle the normal site of the boundary, and others appear to define it from the posterior side. Moreover, most such clones do not interdigitate normally with anterior cells, forming abnormally smooth boundaries, even though such clones should now be transformed into anterior-like cells.

One possible explanation is that the effects of *en/inv* expression on affinity can perdure for some time after the formation of the clone. The effects of this perdurance could be further accentuated if, after loss of *en* and *inv*, ectopic *dpp* expression arose in the clone before any change in affinity. The outgrowths and pattern abnormalities induced in neighboring cells by the ectopic Dpp might 'trap' the *en/inv* cells before they had a chance to intermingle with anterior cells. Indeed, some clones near the A/P only straddle the boundary in the sense that they induce an outgrowth that occupies the site of the normal A/P (see Hidalgo, 1994).

However, it should also be pointed out that clones lacking

en and *inv* can never perfectly mimic anterior cells near the A/P boundary, since such clones lack the late expression of *en* and *inv* normally observed in this region (Blair, 1992). Interestingly, *en*$^-$ *inv*$^-$ clones of anterior origin lying between L3 and L4 do also on occasion show abnormal shapes (Fig. 4E), and in two cases apparently extended into posterior territory (Fig. 4F). In addition, it was reported elsewhere that such clones can reduce the distance between L3 and L4 (Hidalgo, 1994). One possible explanation of these phenotypes is that late loss of anterior *en* and *inv* can disrupt Hh-dependent phenotypes. The signalling-affinity model predicts that any loss of Hh signalling will result in an affinity more resembling that of posterior cells, consistent with the abnormal clone shapes and the occasional violation of the A/P boundary. The reduced distance between L3 and L4 is also consistent with this idea, as a similar phenotype is caused by large posterior *hh*$^-$ clones (Hidalgo, 1994), or reduced activity of Fused, a downstream member of the Hh pathway (Ingham, 1993).

Displacement and growth-based models

Still, there is no proof that the compartmental lineage restrictions depend on cell adhesion or recognition, at least in any simple sense. It has been suggested that compartments constitute regions of regulated growth, and that differences in the rates and pattern of growth, rather than affinity alone, help maintain the lineage boundaries (Karlsson, 1984; see also Lawrence and Morata, 1975; Gubb, 1985). It has been further suggested that compartments are essential for determining when the imaginal disc has reached its mature size; once provided with a complete boundary, cells within each compartment somehow regulate growth so that divisions cease when the compartment reaches the appropriate number of cells.

Interestingly, it was reported recently that when a very large *smo*$^-$ clone lies on the anterior side of the A/P boundary, ectopic, boundary-like gene expression is apparently induced to the anterior of the clone (Chen and Struhl, 1996; diagrammed in Fig. 6A). It has been suggested that this is due to the reduced levels of *ptc* expression observed in such clones; posteriorly secreted Hh, no longer limited by binding to the high levels of Ptc normally observed anterior to the A/P boundary, would diffuse further and signal over a longer range (Chen and Struhl, 1996). We did not observe such distortions of compartment-specific gene expression anterior to our smaller *smo*$^-$ clones (Fig. 3) but, in most of our cases, none or very little of the clone lay in the anterior compartment by late third instar. Let us assume that such distortions are occurring earlier in development, before the movement of the *smo*$^-$ clone into posterior territory. If the new boundary of gene expression now creates a new unit of growth control to the anterior of the clone, the increased growth in that now undersized unit may push the *smo*$^-$ clone to the posterior as the new anterior 'compartment' reaches its final size (Fig. 6A). Alternatively, there may be some intrinsic tendency to straighten the new 'compartment' boundary. In either case, the original A/P lineage restriction on the posterior of the *smo*$^-$ clone would be displaced into the posterior region of the wing and maintained by a difference in selector-driven affinity. Models of this sort must still account, however, for the smooth lineage restriction formed to the anterior of the displaced *smo*$^-$ clone and the fact that only the mutant clone is displaced; both of these are suggestive of some difference in affinity between *smo*$^-$ and wild-type anterior cells.

In a more explicit version of this model, the anterior displacement of *dpp* expression would be the critical factor in such growth control. Evidence suggests that the stripe of *dpp* expression along the anterior side of the A/P boundary sets up a gradient of Dpp protein in the growing disc; levels of this TGFβ-like growth factor would then control anterior-posterior growth and patterning (e.g. Nellen et al., 1996; Lecuit et al., 1996). If that region of *dpp* expression was displaced anteriorly, anterior cells, now adjacent to high levels the Dpp growth factor, might increase their rate of division, while posterior cells, now more distant from high levels of Dpp, might decrease theirs. However, the simple form of this model, based solely on growth rates, seems unlikely. While it is true that some basal level of TGFβ-like signalling is required for proper cell division or survival, cells near to or very distant from the Dpp stripe do not differ significantly in their rates of division or the sizes of clones formed (Burke and Basler, 1996). The slight anterior displacement of *dpp* expression would thus have little if any effect on growth rates. Only if the direction and pattern of growth was altered could it explain the precise movement of an anterior *smo*$^-$ clone into posterior territory.

If anterior distortions in the region of *dpp* expression can move *smo*$^-$ posteriorly, might similar mechanisms play a role in the anterior movement of *en*/*inv*$^-$ clones? Posterior *en*/*inv*$^-$ clones become capable of receiving the Hh signal and thus express ectopic *dpp* (Sanicola et al., 1995; Zecca et al., 1995; Tabata et al., 1995). *en*/*inv*$^-$ clones immediately posterior to the A/P would therefore induce an abnormal extension of the *dpp*-expressing stripe into anatomically posterior territory. This could induce localized changes in the pattern of growth, helping to push the clone into anterior territory.

Vein formation in *smo*$^-$ clones

Whatever mechanism underlies the posterior movement of anterior *smo*$^-$ clones, an explanation is also needed for the apparent ability of such clones to substitute for or replace posterior tissues, despite the anterior-like gene expression in such clones. In particular, the ability of such clones to form vein tissue in the normal position of L4 is striking. Interestingly, recent evidence suggests that Hh can act independently of the Dpp signal to specify the intervein cells between L3 and L4 (Mullor et al., 1997). It is therefore suggested that cells receiving Hh become the L3-L4 intervein cells (in a process that requires the gene *knot*), and that L3 and L4 are formed only in cells that receive no Hh signal but very high levels of a second, Hh-dependent signal (Nestoras et al., 1997; Fig. 6B). While Dpp is a candidate for this Hh-dependent signal, double mutant studies suggest that other unknown factors are sufficient to induce L3-L4 like tissue (reviewed in Nestoras et al., 1997). A displaced *smo*$^-$ clone that neighbors the A/P boundary will be incapable of receiving the Hh signal but will be adjacent to cells producing the Hh-dependent signal. Thus, if it lies just posterior to the boundary, it will form a vein in the position of L4. Similar behavior would also be expected surrounding *smo*$^-$ clones between L3 and L4, as the region producing Hh-dependent signals likely fills this area. Indeed, clones between L3 and L4 often contained ectopic vein tissue.

However, ectopic vein formation cannot be accounted for by proximity to Hh-dependent signals alone. Small *smo*$^-$ clones commonly cause the bifurcation of L3 and L4. One branch

forms within the clone on the side nearest the A/P, and thus nearest the Hh-dependent signal. However, the other branch forms outside the clone on the side most distant from the A/P (Fig. 2G,H). If the Hh-dependent signal is sufficient to trigger vein formation outside the clone, why are veins not formed throughout the clone? In addition, little or no extra venation is observed in cells posterior to large clones on L4, despite the fact that many of these cells are just as close to the A/P boundary as cells to the posterior of small clones. One possibility is that these differences are based on timing. Small clones are formed late in development and may not displace posterior cells until they have already been specified as vein tissue. Large clones are formed earlier in development and may therefore displace posterior cells from the A/P before they have received sufficient cues to form vein tissue. Additionally, the formation of veins involves a process of lateral inhibition between 'pro-vein' cells (de Celis and Garcia-Bellido, 1994), which might lead to the secondary loss of veins from parts of the *smo*$^{-}$ clone.

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