Development 121, 4215-4225 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
DEV7447

Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing

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SUMMARY

Growth and patterning of the *Drosophila* wing is controlled by organizing centers located at the anterior-posterior and dorsal-ventral compartment boundaries. Interaction between cells in adjacent compartments establish the organizer. We report here that Serrate and Notch mediate the interaction between dorsal and ventral cells to direct localized expression of Wingless at the D/V boundary. Serrate serves as a spatially localized ligand which directs Wg expression through activation of Notch. Ligand independent activation of Notch is sufficient to direct Wg expression, which in turn mediates the organizing activity of the D/V boundary.

Key words: Serrate, Notch, Wingless, compartment boundary, pattern formation, wing disc

INTRODUCTION

Pattern formation in multicellular systems depends on short range interactions between distinctly specified cells. Cell interaction across compartment boundaries leads to the formation of pattern organizing centers which control growth and specify cell fates in the leg and wing primordia of *Drosophila* (Diaz-Benjumea and Cohen, 1993; Basler and Struhl, 1994; Diaz-Benjumea et al., 1994; Williams et al., 1994). The first step in establishing an organizing center involves the specification of two differently determined populations of cells in adjacent territories, known as compartments (García-Bellido et al., 1973). Cells are assigned their compartment-specific identity through localized expression of transcription factors (reviewed by Lawrence and Morata, 1994). The homeobox gene *engrailed* and its homologue *invected* function together to specify the posterior compartment of the limb primordia. *engrailed* and *invected* are expressed in all cells of the posterior compartment, where they are required to specify the fate of posterior cells (Sanicola et al., 1995; Zecca et al., 1995). In a similar manner, the dorsal compartment of the wing is established by localized expression of the LIM/homeobox gene *apterous* which specifies dorsal cell fate (Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993; Blair et al., 1994; Williams et al., 1994).

Following the specification of the compartments, an asymmetric cell signal is transmitted at the interface between the two cell populations. Cells in the posterior compartment communicate with nearby anterior cells through expression of the secreted signaling molecule Hedgehog (Hh; Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Basler and Struhl, 1994; Tabata and Kornberg, 1994; Diaz-Benjumea et al., 1994). The Hh signal is transduced through a pathway involving the *patched* and *protein kinase A* gene products (Ingham et al., 1991; Ingham, 1993; Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995), and leads to localized expression of the secreted signaling molecule Decapentaplegic (Dpp) in anterior cells near the compartment boundary of the wing disc. Localized expression of Dpp is thought to specify cell fates and control growth in the developing wing imaginal discs (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Ingham and Fietz, 1995; Zecca et al., 1995). In the leg disc, Hh directs Dpp expression in dorsal cells and Wingless (Wg) expression in ventral cells (Basler and Struhl, 1994), and the combined activity of these two signals patterns the leg (Diaz-Benjumea et al., 1994; Campbell et al., 1993).

A comparable process of short range interaction between dorsal and ventral cells leads to formation of a growth control center at the D/V compartment boundary. This process requires a signal between dorsal and ventral cells which leads to localized expression of a signaling molecule at the compartment boundary. Expression of Wg in a row of cells that straddles the D/V boundary is an early consequence of interaction between dorsal and ventral cells (Couso et al., 1993; Phillips and Whittle, 1993; Williams et al., 1993; Couso et al., 1994). We report here that localized expression of Wg is sufficient to mediate both the cell fate specification and growth control activities attributed to the D/V boundary organizing center. We present evidence that activation of the transmembrane receptor Notch is necessary and sufficient to direct localized expression of Wg at the D/V boundary, and that expression of the Serrate protein in dorsal cells provides a spatially localized ligand for Notch in this process. These findings suggest that the interaction between Serrate and Notch is required to establish the localized domain of Wg expression in the D/V compartment boundary.

MATERIALS AND METHODS

Ectopic expression of Wg, Notch and Serrate

Ubx>f+>wg

A 3 kb *Ubx* enhancer fragment linked to a 2.5 kb fragment of the *Ubx* promoter were used to drive expression of a *wg* cDNA. The *Ubx* enhancer fragment has been shown to direct reporter gene expression throughout the wing disc (Castelli-Gair et al., 1992), although at non-uniform levels in different positions (W. Brook, unpublished data). The flip-out cassette consists of a 5.4 kb genomic fragment of the wild-type *forked*$^{+}$ gene flanked by FRT sites and cloned between the *Ubx* promoter and the *wg* cDNA in a Car 20 vector, as described by Struhl and Basler (1993). The *forked*$^{+}$ genomic fragment used in this construct proved to be unable to rescue the *forked* mutant phenotype in trichomes, but was a reliable marker for bristles. Wg-expressing clones were induced by heat shock treatment at 35°C. Clone frequency varied with the duration of the heat shock.

act5C>y+>Notch

The act5C>y^{+}>Notch (intra) flip out construct is described by Struhl et al. (1993). Notch-expressing clones were induced in larvae carrying a *wg-lacZ* reporter gene or following a cross with *y* HSFLP1.

UAS-Ser

The UAS-Serrate construct is described by Speicher et al. (1994). Flies carrying both UAS-Ser and UAS-βGAL transgenes were crossed with the GAL4 driver lines Ptc-GAL4 or Gal4-459.2 (Speicher et al., 1994; Thomas et al., 1995). Serrate-expressing cells were visualized by X-gal staining and discs were labeled with an antibody to Wg.

Somatic mosaic analysis

Somatic mosaic clones were generated by irradiating larvae with 1000 Rad of X-rays at 72±12 hours after egg deposition. Mutant clones were given a growth advantage using the Minute technique (Morata and Ripoll, 1975). Clones were marked by loss of the cell autonomous bristle and trichome marker *forked*. Details of the genotypes and results are presented in Table 1. Clones of cells mutant for the null allele *sgg*^{D127} (Ruel et al., 1993) were marked in the wing disc by loss of the β-GAL-expressing P-element insertion WG1296 as described by Blair (1992). The relevant larval genotype was *sgg*^{D127}*/WG1296 M(1) o*^{sp}.

Notch temperature shifts

The *Notch* ^{ts1} allele produces a Notch protein that is functional at 18°C but inactive at 29°C (Schallenberger and Mohler, 1978). For temperature shift experiments, eggs were collected for 24-hour periods and larvae raised at 18°C. Larvae were shifted to 29°C when the first mature third instar larvae left the food. Larvae were dissected at 12, 24 and 48 hours after the temperature shift, and stained with an antibody to Wg.

Histochemical methods

Wg antibody was raised by immunizing rabbits with a His-tagged fragment of Wg protein, corresponding to the fragment used by van den Heuvel et al. (1989). Affinity purified Wg antibody was used at a dilution of 1:10. The polyclonal mouse antibody to Dll protein has been described by Vachon et al.(1992). Mouse antibody to Serrate protein (Thomas et al., 1995) was provided by E. Knust and used at a 1:1000 dilution either alone, or following X-gal staining of larvae carrying the apterous-*lacZ* reporter *ap*^{rK568} (Cohen et al., 1992). *sgg* mutant clones were identified by double labeling with rabbit antibody to β-gal and mouse anti-Dll. Samples were examined using the confocal microscope, of the Light Microscopy Group at EMBL.

Table 1. Summary of clonal analyses

Larval genotype	Clones including wing margin			Clones not including margin
	D	V	D+V	
f^{36a}*; wg*^{cx4} *b pr/M(2L)z f*$^{+30B}$	4	7	6	5
f^{36a}*; wg*^{IL114} *cn bw/M(2L)z f*$^{+30B}$	7	5	7	5
f^{36a}*; Ser*$^{rev6.1}$*/f*$^{+87D}$ *M(3R)w*^{124}	12	11	16	7
f^{36a}*; Su(H)*^{AR9}*/M(2L)z f*$^{+30B}$	9	3	4	7

For all experiments clones were induced at 72±12 AEL Minute time, corresponding to second instar. Inducing clones prior to formation of the D/V compartment boundary allows for three outcomes. (1) A clone may cross the boundary, indicated as (D+V). (2) A clone may meet, but not cross, the boundary. Clones that meet the margin on the dorsal side only are indicated D. V indicates clones that meet the margin on the ventral side only. (3) A clone may not meet the boundary at all, indicated under the heading 'clones not including margin'. Whether a given clone crosses the boundary depends on where the cells is the clone were located at the time the boundary was established. If the progeny of the original founder were located such that the clone is bisected by the newly formed boundary, the clone will include both dorsal and ventral margin. Clones that originate close to the nascent boundary, but which are not bisected by it, may subsequently grow along the boundary. Since the clones of mutant cells were given a relative growth advantage in these experiments, clones of this type may include large regions of the wing margin from one side only, as shown in Fig. 2.

Wg: The results of analysis of the two *wg* alleles were identical and are considered together. *wg* clones that meet the boundary from either the dorsal or ventral side do not produce nicks in the wing and only show local perturbation of the structure of the wing margin (*n*=23 clones in total, see Fig. 2 for examples). Clones that cross the D/V boundary produced extensive non-autonomous loss of wing tissue (*n*=13, see Fig. 2). Clones that do not meet the margin produce no phenotype (*n*=10). The number of clones in this class is under-represented, since not all wings were scored for clones that did not include the margin. For the *wg*^{IL114} clonal analysis, larvae were maintained at 18°C following irradiation and were shifted to 29°C at 180 hours AEL.

Ser: *Ser*$^{rev6.1}$ is a null allele described by Fleming et al. (1990). *Ser* clones, which include ventral margin, do not produce nicks in the wing (*n*=11) while clones that include dorsal margin (or both D+V) produce nicks (*n*=28).

Su(H): Clones of cells mutant for *Su(H)* produce nicks if the clone meets the margin from either side (*n*=126 in total). Clones which do not meet the margin show no phenotype (*n*=7 in total). *Su(H)*^{AR9} is a lack of function allele described by Ashburner (1982). Clones of cells mutant for S.

RESULTS

wingless mediates the organizing activity of the dorsal/ventral compartment boundary

The temporal and spatial pattern of *wg* expression in the developing wing imaginal disc prompted us to ask whether the secreted Wg protein could be responsible for mediating the growth control and patterning activities attributed to the D/V boundary (Diaz-Benjumea and Cohen, 1993). The pattern of *wg* expression is dynamic and is thought to reflect distinct functions for *wg* at different stages of development (Phillips and Whittle, 1993; Williams et al., 1993; Couso et al., 1993; Couso et al., 1994). In the second instar wing disc Wg protein is transiently expressed in a ventral anterior domain, which partially overlaps the domain of *apterous* expression in the newly formed dorsal compartment (Williams et al., 1993). *wg* activity is required at this stage for formation of the entire wing (Couso et al., 1993; Williams et al., 1993). Still in the second instar, this domain rapidly changes into a stripe at the presumptive D/V boundary (data not shown). At later stages of development the stripe of Wg-expressing cells induces neighboring cells to differentiate into the sensory bristles that make up the wing margin (Blair, 1993; Phillips and Whittle, 1993; Couso et al., 1994).

In addition to its function in specifying wing margin cell fates, we show here that localized expression of Wg mediates the growth control activity attributed to the D/V compartment boundary. We have used the flip out technique to misexpress Wg. A previously described transgene that used the Actin5C promoter to direct *wg* expression produced relatively low levels of Wg protein and showed little or no effect on wing development (Struhl and Basler, 1993). To ask whether ectopic expression of Wg at higher levels might be more effective, we prepared a construct using a fragment of the Ubx enhancer and promoter (Castelli-Gair et al., 1992). Although clones can be induced in any part of the disc following excision of the flip-out cassette, the level of Wg expression is spatially non-uniform (Fig. 1A).

Clones of Wg-expressing cells in the wing pouch induce the formation of ectopic wing margin structures (Fig. 1B,C). If the clone lies in the dorsal compartment, the ectopic margin consists entirely of dorsal cell types (Fig. 1B) while clones in the ventral compartment produce ventral marginal structures (Fig. 1C). These results indicate that the Wg signal is sufficient to induce cells to adopt wing margin fate, consistent with earlier demonstrations that Wg activity is required for formation of the wing margin (Phillips and Whittle, 1993; Couso et al., 1994). In the endogenous wing margin sense organ precursors are specified in cells adjacent to the Wg-expressing cells, but not in the Wg-expressing cells themselves (Blair, 1993; Phillips and Whittle, 1993; Couso et al., 1994). It is therefore expected that the cells adopting ectopic margin fate need not coincide precisely with the Wg-expressing cells. Nonetheless, the ectopic wing margin bristles are quite often made by genetically marked Wg-expressing cells. This may be due to the fact that most clones express a lower level of Wg than is seen in the endogenous margin (e.g. Fig. 1A).

In addition to inducing the formation of ectopic wing margins, clones of Wg-expressing cells can cause outgrowths from the surface of the wing blade (Fig. 1B,C). Most clones of Wg-expressing cells are located away from the endogenous D/V boundary and so are restricted to either the dorsal or the ventral compartment. The ectopic expression of Wg by these clones promotes local overgrowth of one surface of the wing blade, without affecting cells in the corresponding position on the other surface. The overgrowth forces the epithelial sheet to buckle out perpendicular to the plane of the endogenous wing blade, which may cause folding of the wing as in Fig. 1B or a blister as in Fig. 1C. Since our construct does not mark the trichomes on each wing cell (see Materials and methods for details) we cannot precisely define the border of the clone in these wings. We infer that the effects of Wg-expressing clones in producing outgrowth from the wing surface reflect a non-autonomous influence on the surrounding wild-type wing tissue. This conclusion is supported by the observation that the local effect of the clone is to induce the formation of ectopic wing margin structures in nearby wild-type cells. Thus it seems likely that the long-range effects on growth of the wing reflects a non-autonomous influence of Wg on the wild-type cells in the region surrounding the cells that are induced to form the ectopic wing margin. Consistent with this interpretation, clones of Wg-expressing cells result in a locally graded distribution of Wg protein resembling that seen at the endogenous margin (Fig. 1 D).

An independent line of evidence for a non-autonomous effect of Wg expression in patterning the wing disc comes from examination of the pattern of Wg and Distal-less (Dll) expression. Dll is expressed in a broad band of cells straddling the wing margin (Fig. 1D,E). Experiments using the temperature sensitive allele wg^{IL114} show that Dll expression in this stripe depends on Wg activity at the margin (B. Cohen, data not shown). Clones of Wg-expressing cells induce ectopic Dll expression (Fig. 2D). The domain of Dll expression overlaps the domain of ectopic Wg-expressing cells, and extends several cells beyond where Wg protein can be detected by antibody staining. Although the cells in which the *wg* gene is being expressed are not directly marked in this experiment, the clones of cells expressing the gene are likely to be smaller than the domain in which Wg protein is detected. Thus Wg acts non-autonomously to activate Dll expression in cells near the margin. The effect of Wg on Dll expression is mediated through inactivation of the Sgg/ZW3 kinase. Clones of cells mutant for *sgg/zw3* express Dll in a cell autonomous manner (Fig. 1E). Clones of *sgg/zw3* mutant cells adopt wing margin fate in a cell-autonomous manner but, unlike Wg-expressing clones, they do not affect the growth of the surrounding wing tissue (Simpson et al., 1988; Perrimon and Smouse, 1989; Blair, 1992).

The wing phenotypes described above can be caused by clones induced in the early third instar, after formation of the D/V boundary. On this basis we conclude that the ability of Wg-expressing clones to induce margin fates and promote growth of the wing blade reflects the functions of Wg after the establishment of the D/V compartment boundary (see also below). When clones are induced in first or second instar, we observe an additional phenotype. Clones of Wg-expressing cells can induce the formation of ectopic wing structures in the dorsal thoracic body wall, the notum (data not shown). This suggests that Wg expression may be sufficient to induce body wall cells to adopt wing fate, perhaps reflecting the earlier function of Wg in the second instar wing disc. The ability to induce ectopic wing tissue is not restricted to the wing. Maves and Schubiger (1995) have shown that Wg-expressing clones can transform certain leg tissues to wing tissue.

Localized expression of Wg on either side of the D/V boundary is sufficient to support development of the wing blade

In view of the observation that Wg-expressing clones promote growth of wing tissue, we have re-examined the requirement for Wg activity at the D/V compartment boundary using clonal analysis. Previous studies have led to the conclusion that Wg function is largely non-autonomous since most clones of *wg* mutant cells showed no effect on wing formation, although in rare cases clones caused wing defects (Baker, 1988). We have examined clones of cells mutant for two different alleles of *wg*: the null allele wg^{cx4} and the strong temperature sensitive allele wg^{IL114}. The results of these analyses are comparable (Table 1). In analyzing the effects of mutant clones, particular attention was paid to the position of the mutant cells with respect to the D/V compartment boundary. Clones of mutant cells that cross the dorsal/ventral boundary cause extensive non-autonomous loss of wing tissue (Fig. 2A,B, n=13). In contrast, clones that include either the dorsal or the ventral side of the boundary, but which are restricted to only one side, do not cause nicking of the wing (n=23). An example of a clone

t hat tests the requirement for Wg in the dorsal margin along most of the anterior wing margin is shown in Fig. 2C. For most of its length the clone produces only minor perturbation in the structure of the wing margin (detail in Fig. 2D), however, non-autonomous loss of wing tissue occurs where the clone crosses the D/V boundary, as it does near the distal tip of the wing in this example (Fig. 2C). Although Wg is expressed on both sides of the boundary, these results demonstrate that its activity on either side is sufficient to support formation of the wing.

The requirement for Wg activity examined in these experiments reflects the function of Wg at the D/V boundary and is distinct from the earlier function of Wg, prior to formation of the boundary in the second instar. We have addressed this point by using the temperature sensitive allele wg^{IL114}. Clones of cells mutant for wg^{IL114} were produced in second instar, prior to formation of the D/V boundary. These clones were allowed to grow at the permissive temperature until early-mid third instar, so that the mutant cells retained Wg function until after the D/V boundary was formed. Control animals kept at the permissive temperature produced large clones which showed no mutant phenotype, even when the clones crossed the D/V boundary (data not shown). However, clones that were shifted to the restrictive temperature showed exactly the same range of mutant phenotypes described for the null allele (Table 1).

Taken together, these observations indicate that Wg activity at the D/V boundary is required to promote growth of the wing blade in a non-autonomous manner. Removing Wg causes extensive loss of wing tissue. Providing an ectopic source of Wg protein locally specifies wing margin while exerting a long range influence on growth of the wing blade. The effects of Wg-expressing clones closely resemble those caused when a secondary D/V compartment boundary is induced by producing a clone of *apterous* or of *fringe* mutant cells in the dorsal compartment (Diaz-Benjumea and Cohen, 1993; Irvine

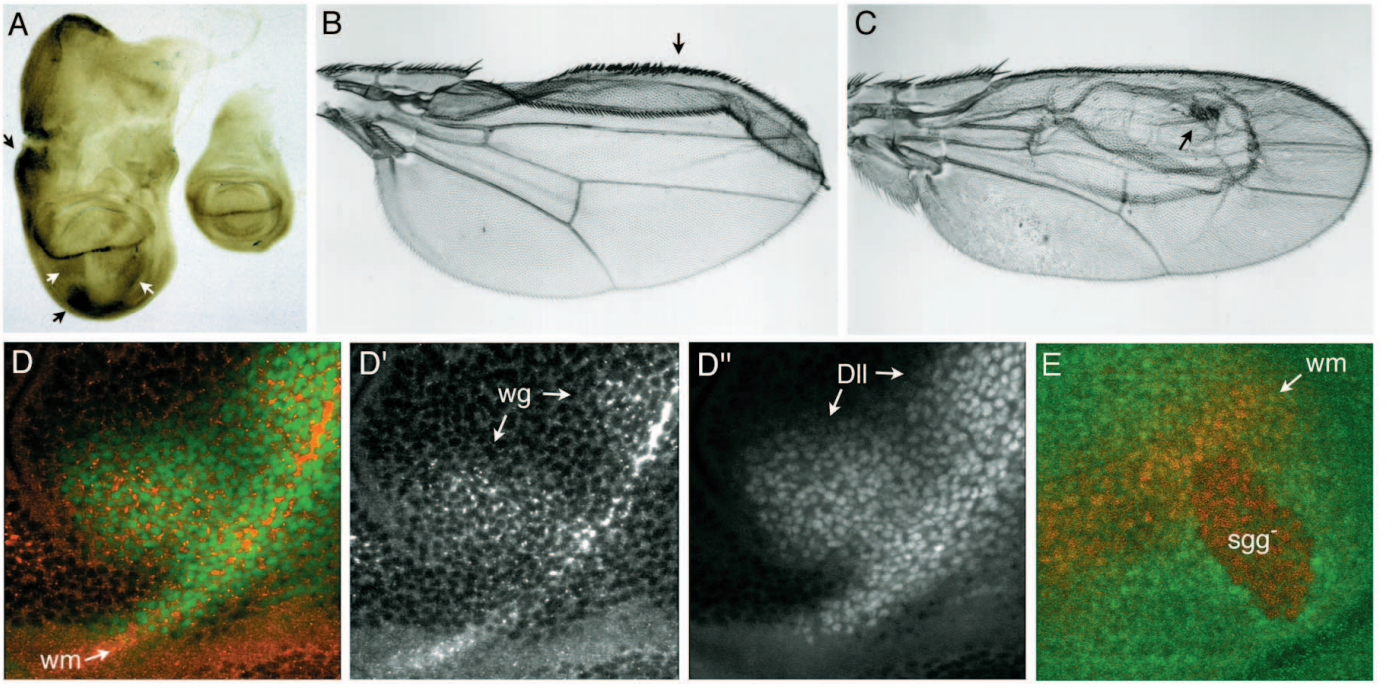

Fig. 1. Organizing activity of Wg protein in the wing disc. (A) Comparison of Wg protein expression in a wing disc with multiple Wg-expressing clones (on the left) and a wild-type sibling disc (on the right). Note the increase in the size of the disc in which Wg is ectopically expressed. Two clones expressing high levels of Wg are indicated by black arrows. Two clones expressing lower levels of Wg are indicated by white arrows. Not all clones are indicated. The vast increase of the notum results in part from transformation to wing tissue, as revealed by expression of the wing pouch-specific marker Nubbin (Ng et al., 1995; data not shown). (B,C) Individual Wg-expressing clones on the dorsal (B) and ventral (C) surface of the wing. The clones produce ectopic wing margin structures (arrows) and cause extensive overgrowth of the surrounding wild-type wing tissue. Since the clones are restricted to one compartment the resulting local overgrowth leads to a buckling of the epithelial sheet on one surface of the wing, without affecting the other surface. The wing in B is seen from the ventral surface. The long narrow clone produces a ridge-like structure which causes the wing to fold (toward the viewer) so that the anterior edge is seen from the dorsal side. The outgrowth projects upward from the plane of the dorsal compartment. The small clone in C produced a more conical outgrowth of the ventral compartment. (D) Non-autonomous activation of Dll by Wg-expressing clones visualized by double immunofluorescent labeling. Dll is expressed in a broad stripe of cells (green nuclei) straddling the domain of Wg expression at the wing margin (wm, red). The single channels for Wg and Dll labeling are shown in D' and D'' respectively. Clones of Wg-expressing cells (arrows in D') induce ectopic expression of Dll (arrows in D''). Dll expression extends several cells beyond where the Wg protein can be detected by antibody staining, suggesting that low levels of Wg are sufficient to activate Dll. (E) Cell-autonomous activation of Dll in a clone of cells mutant for *sgg/zw3*. The Wg signal is thought to antagonize the activity of the Sgg/ZW3 kinase (Siegfried et al., 1992), therefore clones of cells mutant for *sgg/zw3* behave as though they have received the Wg signal (Blair, 1992; Diaz-Benjumea and Cohen, 1994). Dll expression is de-repressed in *sgg/zw3* mutant cells. The clone is marked by the absence of the nuclear β-gal marker WG1296 (green). Dll expression is visualized in red. The overlap of the two labels in the endogenous domain of Dll expression at the wing margin appears orange, while the Dll-expressing nuclei in the clone appear red. The clone includes a portion of the normal Dll expression domain and meets the D/V boundary.

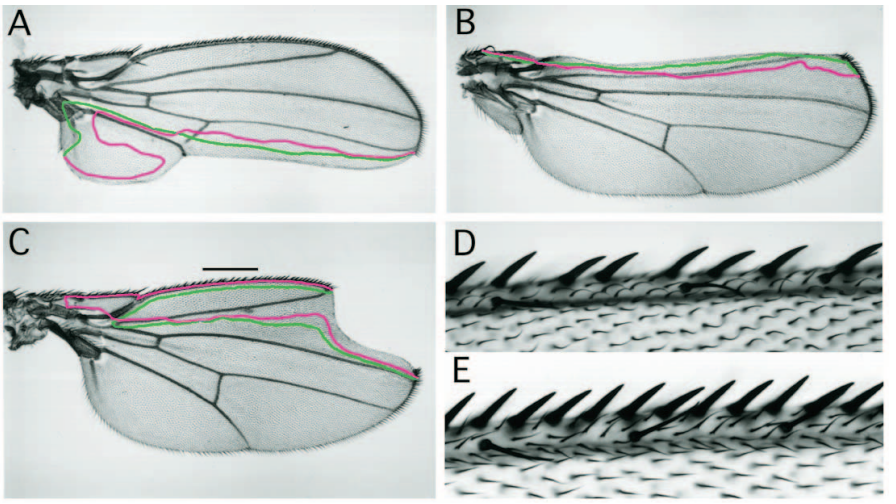

Fig. 2. *wingless* activity in cells on one side of the compartment boundary is sufficient to support wing development. Clones of cells mutant for the null allele wg^{CX4} produce extensive non-autonomous loss of wing tissue. *wg* mutant cells were identified using the cell-autonomous bristle and trichome marker *forked*. The edge of the clone on the dorsal surface of the wing is indicated by the purple line. On the ventral surface the edge of the clone is marked by the green line. All cells between these lines and the near edge of the wing are mutant. Wg is expressed in a row of cells straddling the D/V compartment boundary (Baker, 1988; Phillips and Whittle, 1993; Couso et al., 1993, 1994). (A) A large clone covering part of the dorsal and ventral surfaces of the wing in the posterior compartment. (B) A large dorsal and ventral clone in the anterior compartment. (C) A large anterior clone which includes most of the dorsal wing margin. The clone crosses between dorsal and ventral compartments distally, but does not include the ventral wing margin in most of the anterior compartment. Note that wing tissue is lost only when Wg activity is removed from both sides of the wing margin. (D) Detail of the anterior dorsal wing margin in the region indicated by the bar in C. The bristles and trichomes are mutant for *wg* and the cell marker *forked*. Note that the number of stout mechanosensory bristles of the mutant triple row is reduced in the mutant margin and that they are spaced unevenly. Although these cells are mutant for *wg*, formation of wing margin structures has been largely rescued by Wg expressed in the adjacent ventral cells. Non-autonomous rescue can also be seen near the edges of the *wg* mutant clones in A-C. (E) Detail of a wild-type wing margin for comparison.

and Wieschaus, 1994), suggesting that localized expression of Wg is responsible for the long range growth promoting properties of the D/V boundary, and later in development for the local specification of cells fates in the wing margin.

Notch signaling directs *wingless* expression in the D/V boundary

The *Serrate* and *Notch* genes are required for formation of the wing (Speicher et al., 1994; De Celis and Garcia Bellido, 1994). Dominant mutations in either gene produce a scalloping phenotype resembling that caused by loss of Wg activity at the margin in the third instar (De Celis and Garcia Bellido, 1994; Thomas et al., 1995; Couso et al., 1994). *Serrate* encodes a predicted membrane-spanning protein with extracellular EGF repeats (Fleming et al., 1990; Thomas et al., 1991). Serrate is thought to serve as a ligand for signaling through the membrane-spanning receptor protein *Notch* (Rebay et al., 1991).

Analysis of mutant phenotypes has suggested interactions between Notch and Wg in formation of the wing margin (Couso and Martinez Arias, 1994; Hing et al., 1994). To assess the nature of this interaction we examined Wg expression in *Notch* mutant discs. Wg expression is lost from the wing margin and the size of the wing is significantly reduced when Notch activity is removed during third instar, using the temperature sensitive allele N^{ts1} (Fig. 3A). Wg expression

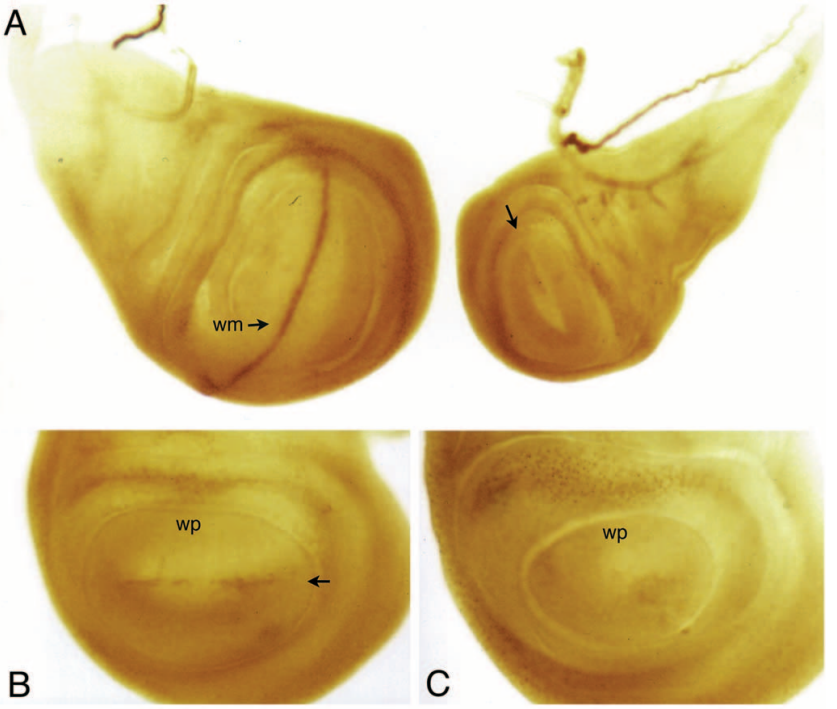

Fig. 3. Notch activity is required for Wg expression at the wing margin. (A) Reducing Notch activity during the third larval instar using the temperature sensitive allele $Notch^{ts1}$ leads to reduction in the size of the wing and to loss of *wg* expression at the wing margin (wm, arrows). The wild-type disc on the left was photographed together with the $Notch^{ts1}$ mutant disc on the right. In discs where residual Wg expression is seen in the margin (arrow in B), the size of the wing pouch (wp) is not reduced as severely as in discs where Wg is completely absent (C). The degree of loss of Wg expression appears to depend on the age of the larva at the time when *Notch* is inactivated.

is reduced, but not completely lost from the margin of larvae within 12 hours of the shift to the restrictive temperature (Fig. 3B), but is absent by 24 hr after the shift (Fig. 3C). The extent to which the wing pouch is reduced, correlates well with the amount of residual Wg expression (compare the tiny wing pouch in Fig. 3C with the moderate sized wing pouch in 3B). Although Notch is expressed throughout the wing disc (Hing et al., 1994), its function in formation of the wing depends only on its activity in cells adjacent to the D/V compartment boundary (De Celis and Garcia Bellido, 1994). Analysis of genetic mosaics shows that the role of *Notch* in activating *wg* depends only on Notch activity in the cells that meet the D/V boundary. Clones of cells mutant for *Notch* fail to express *wg*, if the clone meets the D/V boundary from either side, while comparable clones that do not touch the boundary have no effect on *wg* expression (Rulifson and Blair, personal communication). Taken together these data suggest that Notch activity is required for *wg* expression in the wing margin.

Activation of Notch is sufficient to direct Wg expression

To determine whether N activity activates Wg expression (as opposed to being required for its maintenance), we have made use of a flip-out construct to express the ligand-independent intracellular form of Notch protein (Notch(intra); Struhl et al., 1993). Clones of Notch(intra)-expressing cells cause ectopic expression of Wg in the wing pouch, the region of the disc corresponding to the presumptive wing blade (Fig. 4A-C). The resulting patches of ectopic Wg-expression cause overgrowth of the wing pouch (Fig 4A-C, see above). In the adult wing these clones induce the formation of ectopic wing margin structures and outgrowths from the wing surface (Fig 4D,E), phenotypes which strikingly resemble those produced by ectopic expression of Wg (compare with Fig. 1B,C). Although the clones of Notch-expressing cells are genetically marked with *yellow*, the bristles of the ectopic margins are genotypically wild-type (Fig. 4F,G). This observation suggests that wild-type cells near the clone are induced to adopt wing margin identity. Careful examination revealed patches of unusual cuticle lying within the ectopic margin, often flanked by wing margin bristles (Fig. 4 F,G). These observations suggest that the Notch(intra)-expressing cells are incapable of differentiating into the sense organs that characterize the wing margin, but induce their formation in nearby wild-type cells, presumably via localized expression of Wg. Notch functions in a process of lateral inhibition to specify epidermal as opposed to neural cell fate (Heitzler and Simpson, 1991), consistent with the observation that clones of cells expressing the activated form of Notch are unable to differentiate the sensory bristles of the notum (Struhl et al., 1993). The sense organ precursors of the endogenous wing margin are specified in cells adjacent to the zone of high Wg expression at the D/V boundary (Phillips and Whittle, 1993; Blair, 1993; Couso et al., 1994). Although we were unable to definitively determine the genotype of the cells producing the patches of unusual cuticle using the *yellow* marker, we infer that they are the Notch(intra)-expressing cells.

These observations indicate that the Notch signal is sufficient to direct Wg expression in the developing wing pouch and that the effects of producing clones of activated Notch in

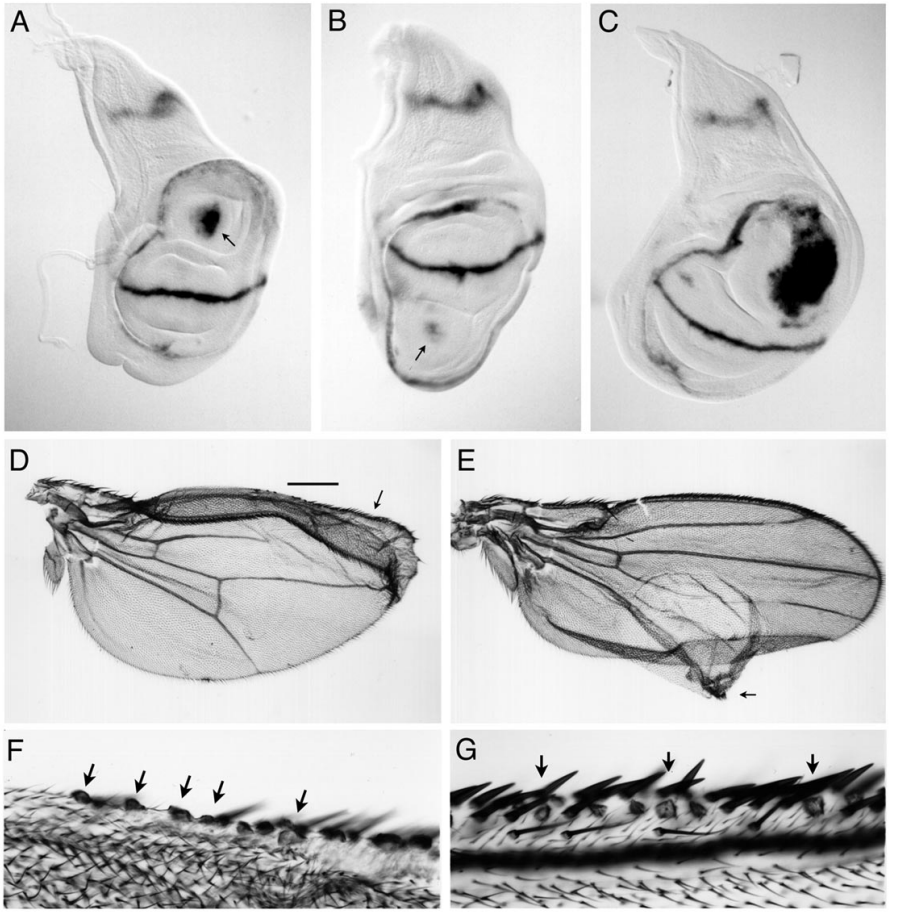

Fig. 4. Notch activation is sufficient to direct Wg expression and to produce bifurcation of the wing. (A-C) Ectopic expression of a *wg-lacZ* reporter gene following ectopic expression of the ligand-independent intracellular form of Notch. (A,C) Clones causing ectopic *wingless* expression in the dorsal compartment leading to duplication of the dorsal wing pouch. (B) Duplication of the ventral wing pouch. (D) Dorsal clone of Notch(intra)-expressing cells producing an ectopic wing margin (arrow). Note the striking similarity of this wing to the dorsal duplication caused by ectopic Wg expression in Fig. 1B. The bar indicates the approximate region magnified in F. (E) Ventral clone of Notch(intra)-expressing cells. Note the similarity to the phenotype produced by a ventral clone of Wg-expressing cells in Fig. 1C. (F) Detail of the ectopic margin in D. Arrows indicate unusual patches of cuticle which may correspond to the clone of cells expressing Notch(intra). These patches are found throughout the ectopic margin. (G) Detail of a different clone of cells expressing Notch(intra). The cuticular structures are nested among the ectopic wing margin bristles (positions of some examples indicated by arrows).

the wing blade are mediated through the observed ectopic expression of Wg. We propose that localized activation of *Notch* in cells adjacent to the wing margin is responsible for directing localized activation of *wg* at the D/V boundary. Consistent with this model, we have found that activity of the *Suppressor of Hairless* (*Su(H)*) gene product is required in the wing margin to support growth of the wing. Su(H) has been implicated as a mediator of Notch-dependent signaling (Fortini and Artavanis-Tsakonas, 1994; Schweisguth, 1995). Clonal analysis using a lack of function allele of *Su(H)* showed that Su(H) activity is needed in cells at the D/V boundary. Mutant clones that include either the dorsal or the ventral wing margin cause non-autonomous loss of wing tissue, while those that do not include the D/V boundary do not cause nicking of the wing (Table 1). These phenotypes are identical to that produced by clones of cells mutant for Notch (De Celis and Garcia Bellido, 1994, and data not shown), consistent with the suggestion that the Notch signal is mediated through Su(H) to direct *wg* expression at the wing margin.

Localized expression of Serrate in dorsal cells provides the signal to activate Wg expression

Three lines of evidence suggest that the Serrate protein may serve as the ligand that activates Notch to direct localized transcription of *wg* at the margin. (1) Serrate expression is restricted to the dorsal compartment of the wing disc during the second and early third larval instars (Fig. 5A,B), when cell interactions establish the D/V organizer (Diaz-Benjumea and Cohen, 1993; Blair, 1993; Williams et al., 1994). (2) Although *Serrate* (*Ser*), is expressed throughout the dorsal compartment, mosaic analysis shows that Ser activity is only required in dorsal cells at the D/V boundary (Fig. 6, table 1). Clones of cells mutant for *Ser* can cause extensive non-autonomous loss of wing tissue, but only when the clone includes the cells that abut the compartment boundary in the dorsal compartment (Fig. 6A). Dorsally located clones that do not include the compartment boundary produce no distinct phenotype (Fig. 6B), nor do mutant clones in the ventral compartment, even if they include the ventral margin (Fig. 6C). (3) Misexpression of Ser in the ventral compartment leads to ectopic expression of Wg (Fig. 5C-E). Wg expression is induced in cells flanking the domain of ectopic Ser expression. However, Wg is not induced to detectable levels in the Ser-expressing cells themselves. These results suggest that only cells that do not express Ser are competent to respond to the Ser signal. Consequently the Ser signal is of necessity acting in an asymmetric manner on cells flanking the domain of Ser expression. The effects of Ser on Wg expression together with the highly localized requirement for Ser activity in cells on the dorsal side of the compartment boundary are compatible with a role for Serrate protein as a ligand transmitting a signal from dorsal to ventral cells.

DISCUSSION

Short and long range influences of Wg at the D/V boundary

Wg is expressed in a stripe along the D/V boundary as a consequence of the interaction between dorsal and ventral cells at the compartment boundary (Diaz-Benjumea and Cohen, 1993; Williams et al., 1994). Wg activity in this stripe is required to specify cell fates in the wing margin in the latter half of the third larval instar (Phillips and Whittle, 1993; Couso et al., 1993, 1994). In addition to its local patterning function we have shown here that Wg activity mediates the long-range patterning effects attributed to the D/V compartment boundary (Diaz-Benjumea and Cohen, 1993). Using somatic mosaic analysis we have shown that Wg activity is required in cells at the D/V boundary to support development of the wing blade. These observations are consistent with the known requirement for Wg activity in controlling expression of downstream effector genes such as *vestigial* and *scalloped*, which are required for growth of the wing (Williams et al., 1993, 1994). Further, we have shown that a localized source of Wg is sufficient to induce wing margin formation and to promote growth of the wing blade. These findings suggest that Wg mediates the organizing activity of the D/V boundary. In this regard the activity of Wg in D/V patterning appears to be analogous to the role of Dpp in A/P patterning (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Diaz-Benjumea et al., 1994; Ingham and Fietz, 1995; Zecca et al., 1995).

While it is clear that a localized source of Wg has long range effects on growth of the wing, this need not imply that these effects reflect a direct local stimulus to proliferation. In the vertebrate limb a signal mediated by localized expression of FGFs in the apical ectodermal ridge promotes growth of the underlying mesenchyme (Niswander and Martin, 1993; Niswander et al., 1993), leading to formation of a localized growth zone (reviewed by Tickle and Eichele, 1994). There are no comparable growth zones centered on either the A/P or D/V compartment boundaries in the developing limb primordia of *Drosophila* (González-Gaitán et al., 1994). Rather, the localized sources of these secreted signaling molecules appear to provide signals which lead to relatively uniform growth throughout the imaginal discs. However, the observation that localized signals elicit de-localized growth responses raises the possibility that some of the long-range patterning activities of Wg and Dpp may be mediated through a signal relay system.

We have shown previously that activating the Wg signal transduction system in a cell autonomous manner is sufficient to elicit all of the long-range patterning activity attributed to Wg in the leg (Diaz-Benjumea and Cohen, 1994). Removing activity of Sgg/ZW3, a cytoplasmic protein kinase, mimics activation of the Wg pathway, so that the mutant cells behave as though they had received the Wg signal (Siegfreid et al, 1992). Our findings demonstrated that at least some of the non-autonomous functions attributed to Wg protein need not depend on the formation of a local gradient of Wg protein, but rather theyare the consequence of a downstream signal relay process (Diaz-Benjumea and Cohen, 1994). Activating the Wg pathway by producing *sgg* clones in the wing mimics the patterning effects of Wg, but in a strictly cell autonomous manner. *sgg* clones turn on Wg-dependent target genes such as *Dll* (this work) or *Vestigial* (Blair, 1992), as well as inducing cells in the clone to differentiate as wing margin sense organs (Simpson et al., 1988; Perrimon and Smouse, 1989; Blair, 1992). However, in contrast to their effects in the leg, *sgg* clones do not promote growth and repatterning of the surrounding wild-type tissue in the wing. This comparison suggests that the long-range non-autonomous effect of Wg in promoting growth of the wing may be mediated through an alternative signaling pathway (see e.g. Hooper, 1994).

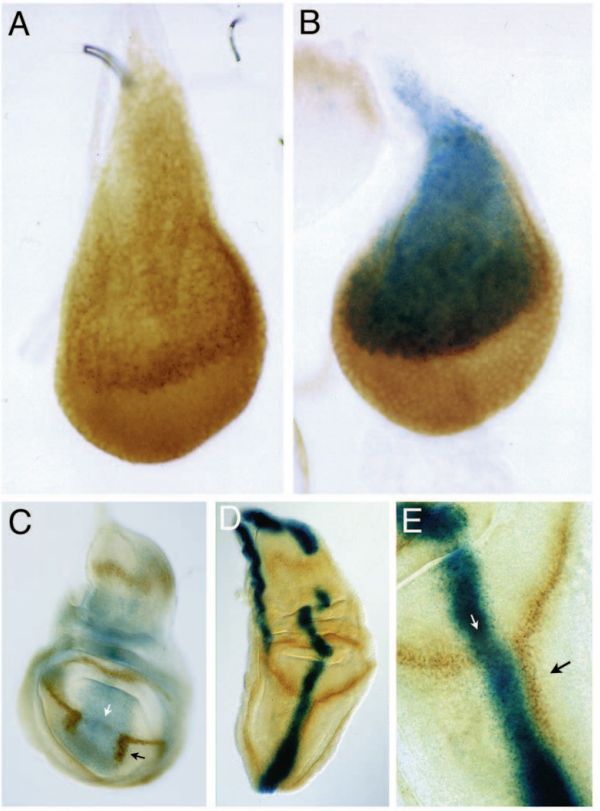

Fig. 5. Serrate expression and misexpression. (A) Serrate protein is expressed in the dorsal compartment of early third instar wing discs. Ser expression can be detected in late second and early third instar discs in the dorsal part of the presumptive wing pouch. Note the elevated level of Serrate near the edge of this domain. (B) Double labeling of Serrate (brown) and a *lacZ* reporter reflecting the expression of *apterous* in the dorsal compartment (blue). The boundary of *ap* expression coincides with the dorsal ventral compartment boundary (Diaz-Benjumea and Cohen, 1993). Note the coincidence between the *ap* domain and the domain of Ser expression. (C-E) Ectopic expression of Serrate in the ventral compartment using the GAL4 system. Larvae carried both UAS-Ser and UAS-β-gal to allow direct visualization of Serrate-expressing cells by X-gal staining (blue). Discs were also labeled with an antibody to Wg protein. (C) UAS-Ser + UAS-β-gal crossed with GAL4-459.2. (D,E) UAS-Ser + UAS-β-gal crossed with Ptc-GAL4. Wg is expressed ectopically in cells flanking the domain of ectopic Ser expression in the ventral compartment (black arrows). Note that WG expression at the endogenous margin is repressed in the Ser-expressing regions (white arrows). These observations indicate that Serrate is able to induce Wg expression in nearby ventral cells, but that Serrate-expressing cells are themselves refractory to this signal. The asymmetric induction of Wg in the posterior compartment in D and E probably reflects low level ectopic Serrate expression throughout the anterior compartment under control of the Ptc-GAL4 driver. As indicated in C there is no inherent bias against activation of Wg in ventral-anterior cells.

Interaction between dorsal and ventral cells directs *wingless* expression at the D/V boundary

We have presented evidence that Ser acts in dorsal cells adjacent to the compartment boundary to provide a localized signal that directs Wg expression at the margin. The properties of Serrate as the signal between dorsal and ventral compartment are strikingly similar to the properties of Hh as the signal between posterior and anterior compartments. Both genes are expressed throughout their respective compartments, though somatic mosaic analysis indicates that their activities are required in cells adjacent to the compartment boundary (this work; Mohler, 1988; Basler and Struhl, 1994). Furthermore, misexpression in the opposite compartment leads to expression of their respective target genes in nearby cells: Serrate directs Wg expression (Fig. 5) while Hh directs Dpp expression (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Ingham and Fietz, 1995). The Serrate signal appears to be mediated through Notch. Notch activity is required for activation of Wg expression (figs 3, 4; Rulifson and Blair, personal communication). We have shown that activation of Notch is sufficient to direct Wg expression and that localized expression of Wg in turn mediates the organizing activities of the D/V boundary. These observations are difficult to reconcile with the proposed role of Notch as a component of the Wg receptor (Couso and Martinez Arias, 1994; Hing et al., 1994), although we have not formally excluded the possibility that Notch might be both upstream and downstream of Wg.

Our findings suggest that expression of Serrate in dorsal cells provides the primary signal to direct localized Wg expression at the compartment boundary (Fig. 7). Like Hh at the A/P boundary, Serrate acts to induce Wg expression in nearby cells. The effects of ectopic expression of Serrate in the ventral compartment reinforce this interpretation. Ectopic Serrate induces Wg expression in nearby cells, while the Serrate-expressing cells are themselves refractory to the Ser signal even though they express Notch (Fig. 5). This is consistent with the observation that Ser does not direct Wg expression throughout the dorsal compartment. Taken together these observations suggest that the primary activation of Wg should be ventral and that a second process of induction leads to Wg expression in the dorsal margin (Fig. 7; although this proposal is inherently testable, the necessary reagents are not yet available).

We can consider two models for the secondary induction of Wg in dorsal cells (outlined in Fig. 7). The two signal model invokes a second signal originating in ventral cells which induces Wg in dorsal cells. Activation of the second signal must depend on the primary signal by Serrate to ventral cells, since Serrate expression in cells on the ventral side of the D/V boundary prevents Wg expression in both ventral and dorsal cells (Fig. 5). However, the second signal cannot depend on Wg function in ventral cells since clones of cells lacking Wg activity at the ventral margin do not preclude Wg expression in the dorsal margin. If dorsal expression of Wg depended on prior ventral expression, ventral-only wg clones would be expected to cause nicks in the margin, but they do not (Fig. 2). This implies the need for two independent responses to the Ser signal in ventral cells: Wg and a second effector required to activate Wg in dorsal marginal cells. Notch is required in both dorsal and ventral cells at the wing margin (De Celis and Garcia Bellido, 1994; Rulifson and Blair, personal communication), suggesting that Notch might also be implicated as a receptor for the second signal. The notion of two separate signals is also supported by the differential activation of the vestigial enhancer in cells on opposite sides of the boundary of an *ap* mutant clone (Williams et al., 1994).

The sequential signaling model uses the primary Ser-

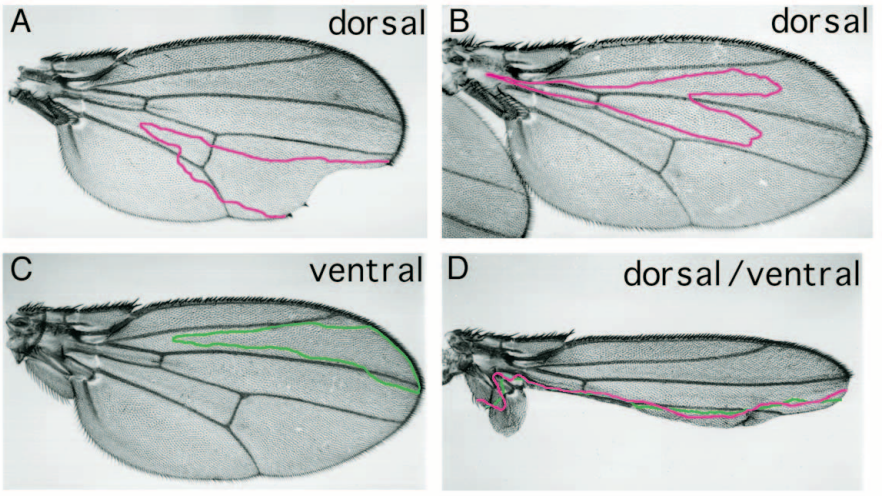

Fig. 6. Serrate activity is required in dorsal cells that abut the compartment boundary. (A) Clone of *Ser* mutant cells in the dorsal compartment. Clones which include the dorsal margin cause loss of wing tissue, resembling that caused by loss of Wg in the margin. (B) Clone of *Ser* mutant cells in the dorsal compartment that does not meet the D/V boundary. Although Serrate is expressed throughout the dorsal compartment the clone causes no loss of wing tissue. (C) Clone of *Ser* mutant cells in the ventral compartment. Although the clone includes the ventral margin there is no nicking of the wing. (D) Large dorsal and ventral clone. Most of the posterior compartment is missing.

mediated signal twice, first to activate Wg in ventral cells and subsequently to activate Wg in dorsal cells. This model is based on the observation that Ser expression precludes cells from responding to Ser by activating Wg. Ser expression is initially coincident with the D/V boundary (Fig. 5), which would lead to expression of Wg in ventral cells. Ser subsequently retracts from the D/V boundary (Speicher et al., 1994), which in principle might allow the Ser signal to activate Wg in dorsal marginal cells. The model is simpler in that it involves fewer components, but requires a mechanism to cause Ser to withdraw from the dorsal margin at a relatively early stage of development. At present we cannot distinguish between the two models.

The relationship between *fringe* and *Serrate*

fringe encodes a predicted secreted protein which has previously been proposed to function as the dorsal signal (Irvine and Wieschaus, 1994). Fringe, like Serrate, is expressed initially in the dorsal compartment of the wing disc and, like Serrate, ectopic expression of Fringe in ventral cells leads to ectopic expression of Wg and formation of ectopic wing margin structures (Irvine and Wieschaus, 1994). Our clonal analysis suggests that Ser has the properties expected of the dorsal signal, in that Ser clones in the dorsal compartment produce no distinct phenotype unless the clone includes the boundary where the signal is transmitted from dorsal to ventral cells. When these clones meet the dorsal boundary, no wing margin is formed and non-autonomous loss of wing tissue results. By contrast, clones of *fringe* mutant cells on the dorsal side of the wing induce the formation of an ectopic wing margin straddling the clone boundary, suggesting that cells lacking Fringe are responsive to the inductive signal from the surrounding cells (Irvine and Wieschaus, 1994). *Ser* mutant clones do not induce ectopic magins and are not responsive to induction by the surrounding cells. Although we cannot definitively rule out a role for Fringe as the dorsal signal, we suggest that the effects of *fringe* clones can be better explained if Fringe renders dorsal cells unresponsive to the Ser signal from the surrounding dorsal cells.

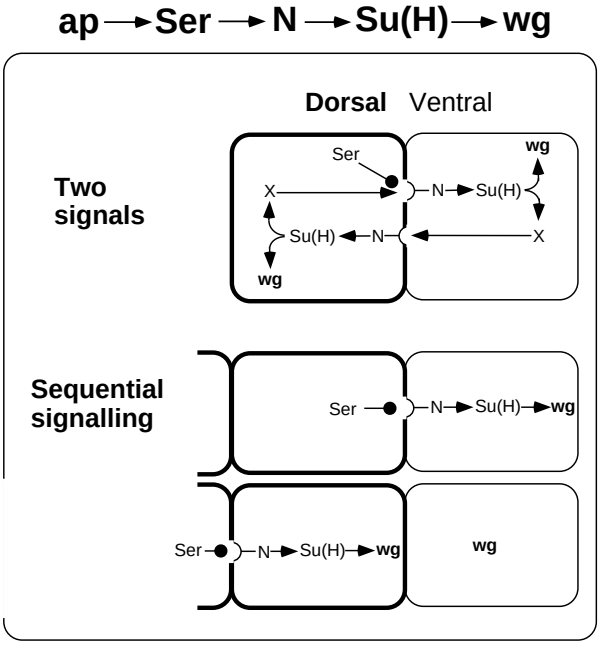

Fig. 7. Models for establishment of the D/V boundary. We propose that Ser is expressed in dorsal cells under control of the dorsal selector gene *apterous* (ap; Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). Ser serves as a localized ligand which signals through Notch to direct Wg expression. Signaling at the A/P boundary is asymmetric. Hh activates Dpp in anterior cells near the compartment boundary (Basler and Struhl, 1994). As outlined in the discussion, we have shown that D/V signaling by Ser is initially asymmetric. Nonetheless Wg comes to be expressed on both sides of the compartment boundary. We present two models that could account for this. The two signal model requires that Ser activate Wg and a second (Wg-independent) response in ventral cells. The second signal feeds back through Notch to activate Wg in dorsal cells. The sequential signaling model requires that Ser and Notch induce Wg sequentially first in ventral cells, and later, after retracting from the D/V boundary in dorsal cells. The rationale for these models is presented in the discussion.

Is the Notch signal instructive or permissive?

The *Drosophila Notch* gene, its homologues, *Lin12* and *Glp1* in *C. elegans* and various vertebrate homologues have been

implicated in a wide variety of cell fate specification events which are effected through local cell interaction (reviewed by Fortini and Artavanis-Tsakonas, 1993; Artavanis-Tsakonas et al., 1995). We have presented evidence that spatially localized expression of the ligand, Serrate, provides an instructive signal for specification of the wing margin organizer. Furthermore, ligand-independent activation of Notch is sufficient to provide the signal, even when the activated form of Notch is expressed in spatially inappropriate positions. These observations suggest that the Serrate/Notch signal is directly instructive. This contrasts with the role of Notch in keeping cells competent to respond to a more specific signal mediated by activation of the sevenless receptor tyrosine kinase (reviewed in Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995), and with the observation that activated Notch prevents cell fate commitment (Coffman et al., 1993; Fortini et al., 1993; Struhl et al., 1993; Rebay et al., 1993). In this sense the function of Ser and Notch at the compartment boundary is perhaps more comparable to that of Lag2 and Glp1 in germ line induction in *C. elegans*, where localized expression of the ligand directs a spatially appropriate response through activation of the receptor (Henderson et al., 1994). The distinction between instructive and permissive signaling through activation of Notch need not imply mechanistic difference in the signaling process, but rather may reflect the context in which the Notch system is being used to convey information between cells.

We thank Mariann Bienz for suggesting the Ubx enhancer/promoter fragment; Elizabeth Knust for providing the antibody to Serrate, the UAS-Serrate and GAL4 driver flies; Gary Struhl for the act5C>y+>Notch(intra) flies; Dirk Lankenau for the *forked* genomic DNA. We are especially grateful to Sandra Scianimanico for devising the affinity purification of the Wg antibody and for generating the Ubx>f+>wg transgenic lines. We thank Alfonso Martinez Arias, Juan-Pablo Couso and Seth Blair for exchanging information prior to publication and Suzanne Eaton for discussion and thoughtful comments on the manuscript.

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(Accepted 13 September 1995)