Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation

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Wing margin formation in *Drosophila* requires the Notch receptor and, in the dorsal compartment, one of its ligands, Serrate. We provide evidence that Delta, the other known ligand for Notch, is also essential for this process. Delta is required in ventral cells at the dorsal/ventral compartment boundary, where its expression is specifically elevated in second-instar wing discs during wing margin formation. Moreover, ectopic Delta expression induces wingless, vestigial, and cut and causes adult wing tissue outgrowth in the dorsal compartment. This effect is mediated by Notch, because loss of Notch activity suppresses Delta-induced ectopic wing outgrowth. Whereas ectopic expression of Notch or the truncated activated Notch induces cut in both dorsal and ventral compartments, ectopic Delta expression induces cut only in the dorsal compartment and ectopic Serrate induces cut only in the ventral compartment. These observations indicate that Notch-expressing cells in a given compartment have different responses to Delta and Serrate. We propose that Delta and Serrate function as compartment-specific signals in the wing disc, to activate Notch and induce downstream genes required for wing formation.

*Key Words:* Delta; Notch; Serrate; *Drosophila*; wing patterning; compartment-specific signaling

Received October 2, 1995; revised version accepted January 17, 1996.

Cell–cell communication is a fundamental process required for patterning and growth during *Drosophila* wing development (for review, see Whittle 1990; Blair 1995). The wing develops from a cluster of undifferentiated cells called the wing imaginal disc. The disc cells are set aside during embryogenesis, proliferate during larval development, and by late third larval instar, the disc has developed the basic pattern elements of the mature wing. Within the epithelial monolayer of the disc, dorsal and ventral compartment cells lie in two adjoining regions (Fig. 2C, below). During pupal development, the dorsal cells become apposed to the ventral cells as the disc folds and everts along the dorsal/ventral (D/V) boundary to form the mature wing blade that is comprised of two symmetrical cell layers. The D/V boundary becomes the wing margin, marked with sensory bristles along the anterior margin and large noninnervated hairs along the posterior margin.

The wing consists of four compartments [anterior, posterior, dorsal, and ventral] as defined by lineage restriction studies (Garcia-Bellido et al. 1973; Blair 1993). Tissue excision and transplantation experiments have demonstrated that juxtaposition of cells from different regions of developing appendages induces proliferation and intercalation of pattern elements (for review, see French et al. 1976). More recently, it has been proposed that interaction between cells with different compartmental identities is required for the normal growth and patterning of the wing and other appendages (Meinhardt 1983; Diaz-Benjumea and Cohen 1993; Tabata and Kornberg 1994).

A number of genes involved in signaling between dorsal and ventral compartment cells have been identified. Dorsal but not ventral cells express *apterous (ap)*, a homeo domain transcription factor that is required for wing disc proliferation and formation of the wing margin [Bourgoin et al. 1992; Diaz-Benjumea and Cohen 1993; Williams et al. 1993]. The *fringe (fng)* gene, which encodes a novel, putatively secreted molecule, is expressed in the dorsal compartment under the control of *ap* [Irvine and Wieschaus 1994]. Juxtaposition of *fng*-expressing and *fng*-nonexpressing cells induce tissue outgrowth and the formation of wing margin structures, suggesting that the *fng* signal can only be received by cells that do not express *fng*. To explain that both *fng*- and *fng*- cells at the borders of *fng*- clones are transformed into wing margin, Irvine and Wieschaus (1994) proposed that *fng* is a dorsal to ventral signaling molecule that induces a reciprocal [ventral to dorsal] signal from the *fng* cells to the *fng* cells. The existence of a reciprocal signal was also proposed by Williams et al. (1994) to explain the similar behavior of *ap* clones.
Candidates for the ventral to dorsal signal include wingless (Wg), a member of the Wnt gene family, which encodes a secreted protein and is essential for wing development, as well as many other developmental processes in Drosophila (for review, see Klingerstsmith and Nusse 1994; Siegfried and Perrimon 1994). Loss-of-function Wg mutations result in abnormal wing phenotypes, ranging from loss of wing margin bristles to complete absence of wing tissue, depending on the particular combination of alleles (Phillips and Whittle 1993; for review, see Lindsley and Zimm 1992). In second-instar wing discs, Wg is expressed in the ventral compartment and may function to maintain the restriction of ap expression to the dorsal compartment (Williams et al. 1993). Both Wg and ap functions are required for the expression of the vestigial (vg) enhancer lacZ reporter construct, the earliest molecular marker for the wing margin (Williams et al. 1994). Later, during third instar, Wg expression is restricted to a stripe at the D/V boundary in cells that later form the wing margin. These results indicate that Wg is required early for proliferation and/or patterning of the disc and later for formation of wing margin structures.

The Notch (N) signaling pathway is also essential for wing development. Loss-of-function mutations in N cause loss of wing tissue similar to that observed in Wg mutants. N encodes a large transmembrane receptor necessary for communication in a number of developmental processes. During neurogenesis, the N gene product, in concert with its ligand Delta (D1), functions to single out neural precursors from fields of neuroectodermal cells (for review, see Campos-Ortega 1988; Ghysen et al. 1993; Artavanis-Takonas et al. 1995). This process requires cell–cell communication among groups of cells, all of which can both send and receive signals (Heitzler and Simpson 1991). Strong genetic interactions between Wg and N indicate that the two genes function in the same pathway during wing margin formation (Couso and Martinez Arias 1994; Hing et al. 1994). Couso and Martinez Arias (1994) have proposed models in which Wg acts upstream or parallel to N during wing margin development and may even be an N ligand; however, loss of N function on either side of the D/V boundary causes loss of wg expression, wing margin, and wing blade tissue in both compartments (dc Celis and Garcia-Bellido 1994; Ruhlison and Blair 1995), suggesting a function for N upstream of wg.

Kim et al. (1995) and Diaz-Benjumea and Cohen (1995) have proposed that Serrate (Ser), an N ligand with sequence similarity to D1 (Fleming et al. 1990; Rebay et al. 1991), functions as a dorsal to ventral signal downstream of fng. Ser expression is restricted to the dorsal compartment in the second-instar wing disc. Loss of Ser function in dorsal cells at the D/V boundary results in loss of wing margin, whereas ectopic expression of Ser in both the dorsal and ventral compartments induces adult wing tissue outgrowth and Wg expression only in the ventral compartment (Speicher et al. 1994; Kim et al. 1995).

The mild wing notching observed with temperature-sensitive combinations of D1 alleles has implicated D1 in wing development, although its role has remained unclear (Parody and Muskavitch 1993). We demonstrate that D1 is required for wing development and can induce a number of genes required for wing formation including Wg, vg, and cut. We propose that D1 encodes a ventral to dorsal signal, because D1 is required in ventral cells at the D/V boundary and ectopic D1 induces cut expression and adult wing outgrowth only in the dorsal compartment. In contrast, ectopic Ser induces cut expression only in the ventral compartment, indicating that N-expressing cells can have different responses to the two N ligands. These results suggest that D1 plays an equivalent but complementary role to Ser as a compartment-specific signal in the genetic program for wing margin development.

**Results.**

**Loss of D1 function during wing development causes loss of wing tissue.**

As part of the N signaling pathway, D1 plays an important role in several developmental processes (for review, see Muskavitch 1994). Using a temperature-sensitive allele of D1, Parody and Muskavitch (1993) have shown that D1 is required during late second and early third instar for wing margin formation. Exposure of such D1 mutant flies to the restrictive temperature at this developmental stage leads to notching at the distal tip of the wing, similar to the phenotype seen in flies heterozygous for an N null mutation. To further analyze the role of D1 in wing development, we generated clones homozygous for a loss-of-function D1 allele, D1^f^ [Heitzler and Simpson 1991], by X-irradiating second-instar larvae. We found that D1 is required for wing margin formation in the ventral but not the dorsal compartment. Ventral clones that abutted the D/V boundary caused gaps in the wing margin (Fig. 1E,F, Table 1), whereas clones that abutted the boundary from the dorsal side and those within the wing blade in either compartment did not cause gaps in the wing margin (Fig. 1C,D). The loss of wing margin phenotype is nearly identical to that caused by clones lacking Ser (Fig. 1B, Speicher et al. 1994; Diaz-Benjumea and Cohen 1995). Clones were identified using forked as a marker [Diaz-Benjumea and Cohen 1993] and by scoring hypertrophy of the wing veins caused by loss of D1 function (Fig. 1C,F, Parody and Muskavitch 1993). Loss of sensory bristles on the anterior wing margin and the noninnervated posterior wing margin hairs was caused by both dorsal and ventral clones that included the domains of cells that give rise to these cuticle structures. All clones, both dorsal and ventral, also caused hypertrophy of vein tissue when they overlapped the normal position of wing veins. The phenotypes in bristle differentiation and wing vein formation are consistent with the known functions for D1 (Parks and Muskavitch 1993; Parody and Muskavitch 1993). The absence of large portions of the wing in D1 mosaics indicates that D1 is required for formation of the wing margin as well as the proliferation and/or viability of wing blade cells. The
Delta in Drosophila wing development

Figure 1. Ventral but not dorsal $D^{pv10}$ clones that abut the D/V boundary cause loss of wing margin. All wings are oriented with anterior up, proximal to the left. Broken lines mark clone boundaries in the dorsal compartment, whereas solid lines mark clone boundaries in the ventral compartment. [A] Unirradiated control $f^{98B}$ wing. The A/P compartment boundary between veins 3 and 4 is marked by an arrow. [B] Wing with a $D^{pv10}$ that crosses the D/V boundary, the anterior wing margin and a large part of the wing blade are missing. Black arrowheads mark groups of forked ebony bristles at the clone borders. (C) Wing with a $D^{pv10}$ clone that abuts the D/V boundary from the dorsal side (bracket), whereas the dorsal marginal hairs are missing and the wing margin and blade are intact. (D) High-magnification view of C. (E) Wing with a $D^{pv10}$ clone that abuts the D/V boundary from the ventral side (bracket); there is a gap in the wing margin and adjacent wing blade. (F) High-magnification view of E.

compartment-specific requirement for DI is the first indication that DI encodes a ventral to dorsal signal reciprocal to the dorsal to ventral Ser signal (Diaz-Benjumea and Cohen 1995; Kim et al. 1995).

Loss of DI function during wing development alters wg expression

Because our mosaic studies indicated that DI plays an important role in wing development, we used wg expression as a marker to examine how loss of DI function in mitotic clones affected formation of the D/V boundary. wg is expressed in the ventral compartment of second-instar wing discs and then along the D/V boundary from early third instar onward (Baker 1988; Couso et al. 1993; Williams et al. 1993). Adult viable wg alleles cause loss of the entire wing and occasional wing to notum transformations (for review, see Lindsley and Zimm 1992); however, reduced wg activity during third instar causes loss of wing margin structures only (Phillips and Whittle 1993).

We generated homozygous $D^{pv10}$ clones in second-instar wing discs and examined wg protein expression in these discs at late third instar using an anti-wg antibody (van den Heuvel et al. 1989). We identified clones by the absence of heat shock-induced expression of a nuclear Myc marker on the $D^{+}$ third chromosome (see Materials and methods). wg expression was altered when mutant clones intersected the D/V boundary. In large clones, we consistently observed a reduction in wg ex-

Table 1. DI is required only in the ventral compartment for wing margin formation

| Location of clone* | Total clonesb | Only dorsal bristles absent | Only ventral bristles absent | Dorsal and ventral bristles absent [no gap] | Gap in margin and blade |
|-------------------|--------------|---------------------------|-----------------------------|--------------------------------------------|------------------------|
| Dorsal            | 11           | 11                        | 0                           | 0                                          | 0                      |
| Ventral           | 10           | 0                         | 2c                          | 0                                          | 8                      |
| Dorsal and ventral| 31           | 0                         | 1d                          | 2                                          | 28                     |
| Internal [dorsal or ventral] | 14          | 0                         | 0                           | 0                                          | 0                      |

*Clone location was determined based on the presence of forked hairs adjacent to the D/V boundary in the dorsal, ventral, or both compartments; forked hairs were confined to the wing blade in internal clones.

bOnly wings with a DI wing vein phenotype were analyzed; six wings with clones were unscorable owing to gross distortion of the wing tissue.

cWings with clones that appear to abut the boundary because they eliminate ventral bristles.

dWing with both dorsal and ventral forked hairs; wing margin and dorsal bristles unaffected.
pression at the D/V boundary in homozygous D1 cells that were surrounded by other homozygous D1 cells (not shown). At the borders of narrower clones, in the homozygous D1 cells that were adjacent to heterozygous D1 cells, D/V boundary wg expression was elevated and ectopic wg expression extended into the ventral compartment (Fig. 2A,B). The separation between wg and Myc expression is attributable to the membrane association of wg and nuclear localization of Myc. Rarely, low levels of ectopic wg expression extended for short distances into the dorsal compartment along the inside border of clones. These results suggest that D1, like fng (Irvine and Wieschaus 1994), can induce wg expression at the border between D1-expressing and D1-nonexpressing cells.

DL expression is elevated at the D/V boundary in second-instar wing discs

The effect of D1 clones on wg expression raises the question of whether D1 plays an early role in setting up the D/V boundary, so we examined D1 expression in wing discs during second and third instar when proliferation and wing margin formation occur. To characterize D1 expression with respect to the dorsal and ventral compartments, we stained discs expressing an ap-lacZ enhancer trap, which marks cells of the dorsal compartment (Diaz-Benjumea and Cohen 1993), with antibodies to β-galactosidase and D1 (Kooh et al. 1993). The earliest patterned D1 protein expression appeared during mid-second-instar. The highest levels of D1 were centered

Figure 2. D1 clones and wild-type D1 expression in the wing disc. [A,B] Homozygous D1 clones in a third-instar wing disc (box in C marks the region of the disc in the images). [A] wg expression (red). The arrow marks the endogenous stripe of wg expression at the D/V boundary, whereas the arrowheads mark ectopic wg expression. [B] Overlay of A with the Myc epitope tag signal (green), the homozygous D1 clones (broken lines) are marked by the absence of Myc signal. Ectopic wg is present approximately one cell width from the nuclei of myc-expressing cells. [C] Fate map of wing disc (Bryant 1975) adapted from Diaz-Benjumea and Cohen (1993); the box marks the region shown in [A,B]. [D–F] D1 protein and ap-lacZ expression in a mid-second-instar wing disc. Dorsal is to the left. Scale bar, 8 μm. [D] ap-lacZ (green) is expressed in the nuclei of dorsal compartment cells. [E] D1 protein (red) is expressed in the membranes of cells at the D/V boundary and mainly in the ventral compartment. [F] Overlay of D and E. [G–I] D1 and wg expression in a wild-type third-instar wing disc. In all wing disc figures anterior is up, dorsal is to the left. Scale bar, 50 μm. [G] Endogenous D1 (green) is expressed in two stripes of cells along the prospective wing margin (white arrows), the prospective wing veins (yellow arrows), and in proneural clusters. [H] Endogenous wg (red) is expressed in the prospective wing margin (white arrow) and in a band across the notum. [I] Overlay of G and H. The wg stripe is flanked by the D1 stripes.
Ectopic expression of Dl results in abnormal outgrowth of dorsal wing tissue

Having found that Dl is required for wing margin formation, we examined whether ectopic Dl expression could induce an ectopic wing margin or wing tissue outgrowth. Using the GAL4 system [Brand and Perrimon 1993], we ectopically expressed a UAS–Dl transgene in the wing disc with the patched-GAL4 enhancer trap line G559.1 [ptcG4] [Hinz et al. 1994]. We refer to the combination of ptcG4 with UAS–Dl as ptcG4–Dl. ptcG4 is expressed strongly in a stripe along the anterior/posterior [A/P] border of the wing disc by mid third instar, with the highest level at the sharp posterior border and gradually lower levels toward the more irregular anterior border [see Materials and methods, Kim et al. 1995]. The GAL4 system has been reported to give more extreme ectopic expression phenotypes at higher temperatures [Speicher et al. 1994]. When raised at 29°C, all of our UAS–Dl lines were lethal in combination with ptcG4. At 22°C, one line [UAS–DI$^{50A1}$] produced viable ptcG4–Dl adults with disrupted anterior cross veins. Shifting ptcG4–Dl$^{50A1}$ larva to 29°C for 24 hr during second instar resulted in adult flies with striking wing outgrowth. The abnormal wing tissue outgrowth occurred only on the dorsal side of the wing blade [Fig. 3A–C]; patches of large bristles characteristic of the anterior double row or posterior wing margin were present at the distal tip of each outgrowth. We found even more extreme outgrowth in the wings of ptcG4–Dl$^{50A1}$ pharate adults raised at 29°C throughout development. Other UAS–Dl insertions crossed to ptcG4 yielded pharate adults with extreme wing outgrowth even when raised at 22°C. These pharate adults also displayed severe defects in the legs, heads, nota, and male genitalia. Thus, ectopic expression of Dl can induce wing outgrowth and a new wing margin, as well as defects in other tissues.

Ectopic Dl acts through N

Dl has been shown to signal through the N receptor during neuronal precursor selection, and this signaling is sensitive to levels of both N and Dl [Vaessin et al. 1987; Heitzer and Simpson 1991]. To determine whether ectopic Dl also utilizes the N signaling pathway for induction of wing tissue outgrowth, we asked whether outgrowth depended on wild-type N function. We used the N$^{ts}$ mutation to reduce N activity, raising the temperature to 29°C for 24 hr during late second- and early third-instar larval development resulted in notching of the wing characteristic of the N mutant phenotype [Fig. 3D; Shellenger and Mohler 1978]. Shifting second-instar ptcG4–Dl male larvae that carried the N$^{ts}$ mutation to 29°C for 24 hr suppressed completely the wing outgrowth phenotype, whereas control ptcG4–Dl flies that were N$^+$ or heterozygous for N$^{ts}$ displayed a strong outgrowth phenotype [Fig. 3C].

Further evidence that ectopic Dl functions through N is that ectopic N expression also induces hairs characteristic of the wing margin, similar to the phenotype of ptcG4-Dl flies raised at 25°C. We ectopically expressed wild-type UAS–N [gift from P. Simpson, Institute of Genetics and Molecular and Cellular Biology, Centre de Recherche National Scientifique (CNRS), Strasbourg, France] by crossing it to ptcG4 [ptcG4–N]. ptcG4–N flies raised at 29°C throughout development had a short row of ectopic hairs along the fourth wing vein on both the dorsal and ventral sides of the wing blade, reminiscent of anterior double row or posterior wing margin hairs [Fig. 3E,F]; these flies occasionally displayed mild wing tissue outgrowth [data not shown]. Taken together, these data confirm that ectopic Dl acts through N, its known receptor, to induce abnormal wing outgrowth.

Ectopic expression of Dl induces wg, vg, and cut expression

Our mosaic studies indicated that Dl may be able to induce wg expression at the border between Dl-expressing and Dl-nonexpressing cells. To examine this possibility further, we looked for ectopic wg expression in third-instar ptcG4–Dl wing discs. wg was induced mainly along the posterior border of the ectopic Dl stripe and rarely at lower levels along the anterior border. In the dorsal compartment, wg was induced in the cells just inside and just outside the posterior border of the ptcG4–Dl stripe, whereas in the ventral compartment, wg was induced at a lower level and only outside the stripe [Fig. 4A–C]. Induction of wg was not limited to lines that expressed GAL4 at the A/P boundary; lines driving UAS–Dl expression at other locations within the wing pouch also induced wg expression and disc tissue outgrowth but not adult wing outgrowth [data not shown]. We did not observe wg induction outside the wing pouch, suggesting that there are region-specific factors that modulate the ability of Dl to induce wg expression.

In third-instar wing discs, ectopic Dl expression also induced cut, vg, deadpan [dpm], and big brain [bib] in cells along the ptcG4 stripe [Fig. 4D,E; additional data not shown]. Whereas all four of these genes are normally expressed in the prospective wing margin [Williams et al. 1991; Bier et al. 1992, Blochlinger et al. 1993; D.
Doherty et al.

**Figure 3.** Ectopic wing margin formation and wing tissue outgrowth induced by ectopic D1 is suppressed by Ntl. A) Wing from a ptcG4–D1 fly shifted to 29°C for 24 hr during the late second and early third larval instars. Wing outgrowth with hairs characteristic of the wing margin always occurs on the dorsal side of the wing (bracket). [B] High-magnification view of A, showing the hairs at the distal tip of the outgrowth. [C] Wing from a N\(^{+/+}\); ptcG4/+; UAS-D1/+ female shifted to 29°C for 24 hr during the late second and early third larval instars. Note the D1-induced outgrowth with hairs characteristic of the wing margin at the tip (bracket). [D] Wing from a N\(^{+/+}\); ptcG4/+; UAS-D1/+ male shifted to 29°C for 24 hr during the late second and early third larval instars. The D1-induced ectopic outgrowth is completely suppressed (cf. C). Notches in the distal wing (arrowheads) are due to the reduction in N activity [see text]. [E] Wing from a ptcG4–N fly shifted to 29°C for all of the second and third larval instars. Ectopic margin-like hairs along vein 4 (bracket) occur on both the dorsal and ventral sides of the wing blade. [F] High-magnification view of E, showing ectopic hairs characteristic of the wing margin (bracket).

Doherty, in prep., only cut and vg have wing phenotypes (for review, see Lindsley and Zimm 1992). There was a notable difference in the ectopic expression of cut, which was induced only in the dorsal compartment of the wing pouch (Fig. 4E; Table 2), whereas vg, bib, and dpn, like wg, were induced in both the dorsal and ventral compartments (Fig. 4A–D; data not shown). Furthermore, these discs were distorted by tissue overgrowth in both the dorsal and ventral compartments (cf. Fig. 4A–C with Fig. 2G–I); however, adult wing outgrowth was exclusively dorsal (Fig. 3A–C). Our observations indicate that many genes, including wg, vg, and cut, can be downstream targets of D1 in the wing disc and that D1 has different effects in the dorsal and ventral compartments.

Because ptcG4–D1 wing discs displayed such striking outgrowth, we asked whether compartmental organization was disrupted. We examined expression of *cubitus interruptus*, a gene expressed in the anterior compartment, *engrailed*, a gene expressed in the posterior compartment, and *ap–lacZ*, an enhancer trap expressed in the dorsal compartment, to determine whether ectopic DI expression causes general reorganization of the disc. We found that despite the dramatic dorsal and ventral disc tissue outgrowth associated with ectopic DI expression, both A/P and D/V compartmental organization appeared undisturbed (data not shown). Our results indicate that D1 is able to induce many of the aspects of the normal wing margin program including wg, vg, and cut expression, as well as wing tissue outgrowth and wing margin bristle formation.

**Ectopic expression of N induces wg expression**

We examined ptcG4–N wing discs to determine whether ectopic N expression induced wg and cut in a manner consistent with its role as a receptor for D1. Immunohistochemical labeling of ptcG4–N third-instar wing discs with anti-N and anti-wg antibodies revealed induction of wg expression in a short stripe perpendicular to the D/V boundary that gives rise to the wild-type wing margin (white arrowheads in Fig. 4F). ptcG4–N induced wg and cut in many fewer cells than ptcG4–D1. wg and cut induction occurred in both dorsal and ventral cells but only close to the prospective wing margin (white arrowheads in Fig. 4F,H). Whereas it is formally possible that the levels of N activity were not sufficiently high to induce wg along the entire ptcG4–N stripe, a more likely explanation is that the ectopically expressed N receptor
Figure 4. Ectopic D1 or N expression induces wg, cut, and vg in the third-instar wing disc. Anterior is up and dorsal is left in all images. (A-E) D1, wg, vg, and cut expression in a ptcG4-D1 third-instar wing disc. Ectopic D1 induces wg, vg, and cut expression as well as overgrowth of the dorsal and ventral wing pouch. UAS-D1 was expressed using the ptcG4 enhancer trap in larva raised at 29°C. (A) D1 (green) is expressed ectopically in a stripe along the A/P compartment boundary (white arrowheads). Endogenous D1 expression is barely visible flanking the D/V boundary at this contrast setting. (B) wg (red) is induced ectopically in a stripe along the A/P compartment boundary (white arrowheads). Endogenous wg expression is visible in the D/V compartment boundary cells (white arrow). (C) Overlay of A and B. (D) vg (red) is induced ectopically in a wide band centered on the A/P compartment boundary (white arrowheads). Endogenous vg expression is visible in a wide band of cells centered on the D/V boundary (white arrow). (E) cut (red) is induced ectopically in a stripe along the A/P compartment boundary only in the dorsal compartment (white arrowhead). Asterisks (*) mark the A/P boundary in the ventral compartment that is devoid of cut expression. (F-I) N, wg, and cut expression in a ptcG4-N third-instar wing disc. Ectopic N induces wg and cut expression. UAS-N was expressed using the ptcG4 enhancer trap in larva raised at 29°C. (F) wg (red) is induced ectopically in cells along the A/P compartment boundary only near the prospective wing margin (white arrowheads). Ectopic wg is expressed in cells on both the dorsal and ventral sides of the wing margin. Endogenous wg expression is visible in the wild-type wing margin (white arrow). (G) N (green) is expressed ectopically in a stripe along the A/P compartment boundary (white arrowheads). The yellow arrowhead marks ptcG4-N expression in the peripodial membrane. (H) cut (red) is induced ectopically only in cells along the A/P compartment boundary near the wing margin (white arrowheads). Ectopic cut is expressed in cells on both the dorsal and ventral sides of the wing margin. Endogenous cut expression is visible in the wild-type wing margin in the same cells that express wg (white arrow). (I) Overlay of G and H. cut is expressed within the ptcG4-N stripe. There is only a slight overlap (yellow) because N is a transmembrane protein, whereas cut is nuclear.

was only activated in the cells that are in contact with cells near the wing margin that express D1 and Ser, known ligands for N (yellow arrow in Fig. 2G). In other words, endogenous D1 and/or Ser may activate the ectopically expressed N in the ptcG4-N stripe, inducing wg and cut expression.

*wg is induced at borders between D1 expressing and nonexpressing cells*

In ptcG4-D1 wing discs, D1 was ectopically expressed at a high level in a stripe several cells wide and endogenous N is expressed throughout the disc; however, wg was not induced throughout the ptcG4-D1 stripe. In the ventral compartment, wg was induced mainly in the cells adjacent to the posterior border of the stripe (Fig. 4A–C), indicating that D1-expressing cells do not receive the D1 signal. In the dorsal compartment, wg was induced in cells along the posterior border of the ptcG4-D1 stripe, in cells outside as well as within the stripe. One explanation for wg induction within the stripe is that D1 induces
Table 2. Effects of ectopic D1 and Ser expression in the dorsal and ventral compartments

|                      | ptcG4–Dl | ptcG4–actN | ptcG4–Ser* |
|----------------------|----------|-----------|-----------|
| Dorsal gene expression |          |           |           |
| wg                   | + +      | + +       | - (2)     |
| vg                   | + +      | + +       | - (2)     |
| cut                  | + +      | + +       | - (3)     |
| disc growth          | + +      | + +       | - (1) (2) |
| adult outgrowth      | + +      | N.D.      | - (1) (2) |
| wing margin          | + +      | N.D.      | - (1) (2) |
| Ventral gene expression |        |           |           |
| wg                   | +        | + +       | + + (2)   |
| vg                   | + +      | + +       | + + (2)   |
| cut                  | -        | + +       | + + (3)   |
| disc outgrowth       | + +      | + +       | + + (1) (2) |
| adult outgrowth      | -        | N.D.      | + + (1) (2) |
| wing margin          | -        | N.D.      | + + (1) (2) |

*Not determined because ptcG4–actN causes early pupal lethality.

One possible explanation for these observations is that D1 autonomously inhibits the ability of a cell to receive Dl signal from other cells. To determine whether within the stripe can inhibit Dl signal reception by that same cell, we coexpressed N and D1 using ptcG4–Dl discs, but it was no longer induced in cells outside the stripe (Fig. 4E). Restriction of wg induction to the posterior edge of the ptcG4–Dl stripe cannot be attributable to factors specific to the A/P boundary, because we also observed wg induction at the borders between Dl-expressing and -nonexpressing cells generated by GAL4 lines expressed at other locations.

One possible explanation for these observations is that D1 autonomously inhibits the ability of a cell to receive Dl signal from other cells. To determine whether increasing the level of N would allow Dl-expressing cells to receive Dl signal, we coexpressed N and D1 using ptcG4. cut was expressed throughout the width of the ptcG4 stripe in these discs (Fig. 5A–C), indicating that N was activated by Dl throughout the stripe. cut expression was still restricted to the dorsal compartment, as in ptcG4–Dl discs, but it was no longer induced in cells outside the ptcG4 stripe. It is possible that N expressed within the stripe binds most of the Dl within the stripe, preventing signaling to the adjacent cells that express N at a lower level. These results provide evidence that Dl within a cell can inhibit Dl signal reception by that same cell and that the ratio of Dl to N within a cell may determine its ability to both send and receive the Dl signal.

D1 and Ser have different signaling abilities in the dorsal and ventral compartments

Dorsal and ventral cells respond differently to ectopic D1 expression [Table 2]. Dl induces higher levels of wg in the dorsal compartment than in the ventral compartment, and cut expression and adult wing outgrowth are restricted to the dorsal compartment. Even when high levels of Dl and N are coexpressed in the ventral compartment, cut expression and adult wing outgrowth are not induced in the ventral compartment [Fig. S5A–C; data not shown]. Similarly, dorsal and ventral cells respond differently to ectopic Ser expression [Speicher et al. 1994; Kim et al. 1995; Table 2]. Ser-induced wg, cut, and vg expression, as well as both disc and adult wing outgrowth, are restricted to the ventral compartment [Kim et al. 1995; Table 2, data not shown]. Ser can partially substitute for Dl during neurogenesis in the embryo [Gu et al. 1995], and Dl and Ser have been shown to bind the same EGF repeat in N [Rebay et al. 1991]; thus the Dl-induced wg and vg expression in the ventral compartment may indicate that Dl can partially substitute for Ser. Nonetheless, ectopically expressed Dl and Ser have strikingly different effects in the dorsal and ventral compartments.

Discussion

We have shown that Dl is required during Drosophila wing development as a ventral to dorsal signal. Mitotic clones lacking Dl that include cells on the ventral side of the D/V boundary cause loss of wing margin and blade tissue in both compartments, and Dl protein is elevated at the D/V compartment boundary of second-instar larval wing discs. Furthermore, ectopic Dl expression induces wing margin formation and wing tissue outgrowth. In light of these findings, we discuss the role of other genes in the N signaling pathway, the likely involvement of A/P boundary signaling components, and the requirement for a border between signaling and receiving cells at the D/V boundary during normal wing development. We also propose a model for Dl and Ser function during the early steps of wing margin formation in the second-instar wing disc.

The Dl–N signaling pathway is required for wing formation

The Dl–N pathway consists of a cassette of genes that functions to transmit signals between cells at many stages during development [Jan and Jan 1993, Artavanis-Tsakonas et al. 1995], and we have shown that Dl plays an essential role in wing development, probably by acti-
Figure 5. cut expression in ptcG4-Dl + N and ptcG4-activated N third-instar wing discs. (A–C) Dl, N, and cut expression in a ptcG4-Dl + N third-instar disc. Ectopic N and Dl together still induce cut expression only in the ventral compartment. UAS-Dl and UAS-N were expressed using the ptcG4 enhancer trap in larvae raised at 29°C. (A) Dl [blue] and N [green] are expressed ectopically in a stripe along the A/P compartment boundary (white arrowheads). (B) cut [red] is induced ectopically within the ptcG4 stripe only in the dorsal compartment (white arrowheads). Asterisks [*] mark the A/P boundary in the ventral compartment that is devoid of cut expression. (C) Overlay of A and B. Ectopic Dl, N, and cut expression overlap in the dorsal compartment (white signal) but not in the ventral compartment (blue signal). (D–F) N and cut expression in a ptcG4-activated N third-instar disc. Ectopic-activated N induces cut expression in the dorsal and ventral compartments. UAS-activated N was expressed using the ptcG4 enhancer trap in larvae raised at 16°C. (D) activated N [green] is expressed ectopically in a stripe along the A/P compartment boundary (white arrowheads). (E) cut [red] is induced ectopically within the ptcG4 stripe in both the dorsal and ventral compartments (white arrowheads). Endogenous cut expression is visible at the D/V boundary (white arrow). (F) Overlay of D and E. Ectopic-activated N and cut expression overlap in the dorsal and ventral compartments.

We propose that in addition to N, Dl, and Ser, other genes in the cassette are likely to be involved in wing development. Suppressor of Hairless [Su(H)] is downstream of N in the signaling pathway, and Su(H) protein has been shown to translocate from the cytoplasm to the nucleus upon activation of N by Dl in transfected S2 cells [Fortini and Artavanis-Tsakonas 1994]. Consistent with the requirement for N in wing margin formation and growth, decreased Su(H) function results in a small third-instar wing pouch [Schweisguth and Posakony 1992] and very small adult wings, reminiscent of vg mutant wings [Ashburner 1982]. Su(H) activity is antagonized by Hairless [H], which encodes a novel nuclear protein [Bang and Posakony 1992]. Loss of H function leads to the opposite phenotype, an abnormally large wing pouch [Bang et al. 1991]. Thus, it appears that multiple elements of the Dl–N signaling pathway operate during wing margin formation.

vg has been shown to interact with N during wing margin formation, indicating that these two genes function in the same pathway [Couso and Martinez-Arias 1994; Hing et al. 1994]. Couso and Martinez-Arias [1994] proposed several molecular models to explain this interaction, favoring a model in which N functions as a receptor for vg. The observations that N is required for vg expression [Rulifson and Blair 1995] and that ectopic N and Dl induce vg demonstrate that vg is a downstream target of the Dl–N signaling pathway; however, we cannot eliminate the possibility that vg also functions upstream of N earlier during wing development.

Adult wing outgrowth requires factors expressed at the A/P compartment boundary

Our ectopic Dl expression experiments indicate that Dl can be sufficient to induce wing margin formation. We have strong evidence that the effects of ectopic Dl expression reflect the function of Dl during wild-type development. The UAS-Dl construct produces protein that is localized to the plasma membrane and cytoplasmic vesicles in a manner indistinguishable from wild-type Dl, and UAS-Dl rescues the Dl loss-of-function neurogenic phenotype in the embryo when driven by hairy-G4 [see Materials and methods]. Furthermore, the ectopic expression phenotype is opposite to the loss-of-function phenotype and requires normal activity of N, the only known receptor for Dl.

We have found that ectopic expression of Dl near the A/P compartment boundary results in adult wing tissue outgrowth. Ectopic expression at other locations in the wing pouch causes ectopic gene expression and outgrowth of wing disc tissue but not adult wing tissue out-
growth. Dl-induced adult outgrowth is always associated with wing margin structures, indicating that disc tissue overgrowth may resolve unless it is maintained by an established wing margin. Distal outgrowth caused by fng^- clones also occurs only near the A/P boundary (Irvine and Wieschaus 1994). It is therefore likely that factors specific to the A/P border are required for induction of an ectopic wing margin and the resulting wing outgrowth. One candidate is decapentaplegic (dpp) that is expressed along the A/P border (Posakony et al. 1990). Ectopic dpp expression induces growth and pattern duplication in the wing (Capdevila et al. 1994). Moreover, overlapping expression of wg and dpp in the leg disc is required for proximodistal growth (Struhl and Basler 1993). Ectopic Dl-induced growth cannot be simply due to the ectopic expression of wg in dpp-expressing cells along the A/P boundary, because neither Dl-induced wg expression in the ventral compartment nor ptcG4-driven wg expression in both compartments is sufficient to cause adult wing outgrowth (Fig. 3A, E. Wilder, pers. comm.). It appears that the constellation of ectopic gene expression induced by ptcG4-Dl acts in concert with endogenous factors at the A/P compartment boundary to cause ectopic wing outgrowth. It will be interesting to determine how endogenous factors at the D/V and A/P boundaries interact to cause growth of the wing.

Dl can inhibit signal reception by N in the same cell

Ectopic Dl expression induces wg and cut only at the borders between Dl-expressing and -nonexpressing cells and not in all cells that express ectopic Dl. One possible explanation for this observation is that Dl inhibits N receptor activity when expressed within the same cell as N. Irvine and Wieschaus (1994) have proposed an analogous model for fng and its putative receptor. In our model, the ratio of Dl to N within a cell would determine its ability to receive a signal via N. Signaling would only occur when cells with a Dl/N ratio low enough to allow signal reception are juxtaposed to Dl-expressing cells. This model explains why there is decreased Dl signaling within the ptcG4-Dl stripe. We have tested three predictions of this model: [1] Signaling should be strongest between cells that express high levels of Dl and cells that express low levels of Dl. We observe maximal wg and cut induction immediately posterior to the ptcG4-Dl stripe, where cells expressing high Dl levels are juxtaposed to cells expressing low levels. [2] Increasing the level of N should relieve the Dl-mediated inhibition of N receptor activity. As expected, in discs expressing N and Dl under the control of ptcB4, cut is expressed throughout the width of the ptcG4 stripe. [3] Expressing high levels of Dl should mimic reduction in N function. This prediction is met by two paradoxical observations. Dl overexpression in the wing blade results in hypertrophy of wing veins (Fig. 3A-C), and Dl overexpression in the proneural cluster results in the development of extra sense organs, both of these phenotypes are similar to those caused by reduction in N or Dl activity (Parks and Muskavitch 1993, de Celis and Garcia-Bellido 1994; Fig. 1C-F; D. Doherty and G. Feger, unpubl.).

Dl could exert its inhibitory effect on N activity by directly interacting with N or by indirectly inhibiting N activity via other proteins. Alternatively, Fehon et al. (1990) proposed that Dl in receiving cells might interfere with N signaling by binding Dl ligand on the signaling cells. It should be noted that inhibition of signal reception by Dl does not appear to play a role in the early D/V patterning of the wing, because we did not detect a sharp border between Dl-expressing and -nonexpressing cells; however, later, the wg stripe is flanked on both sides by Dl-expressing cells, and signaling from Dl-expressing to -nonexpressing cells may be important.

A model for symmetrical gene activation at the D/V boundary

N activity is required on both sides of the D/V boundary for wing margin formation, whereas Dl and Ser are each required only on the ventral and dorsal sides, respectively. In addition, cells in the dorsal and ventral compartments respond differently to ectopically expressed Ser and Dl, whereas cells in both compartments respond equally to ectopically expressed activated N (Table 2). One possible explanation for the different activities of Dl and Ser could be their roles as compartment-specific signals. Bidirectional signaling between dorsal and ventral compartment cells has been invoked as a mechanism to generate the wing margin and symmetric growth of the wing (Irvine and Wieschaus 1994; Williams et al. 1994; Rulifson and Blair 1995). Compartment-specific signaling can be generated by spatial restriction of the ligand or by spatial restriction of the response. For example, fng and Ser are expressed only in the dorsal compartment of second-instar wing discs (Irvine and Wieschaus 1994; cited in Kim et al. 1995). Furthermore, Ser is ectopically induced at the borders of fng^- clones in the dorsal compartment, and ectopic fng induces Ser in the ventral compartment; however, ectopic Ser does not induce fng, indicating that Ser functions downstream of fng. Irvine and Wieschaus (1994) have proposed that fng encodes a compartment-specific dorsal to ventral signal, and Kim et al. (1995) have shown that fng-expressing dorsal cells can recognize when they are adjacent to cells not expressing fng and respond by activating expression of Ser, which they propose encodes a dorsal to ventral signal. The existence of a reciprocal signal from ventral to dorsal cells is based on the observation that cells both inside and outside fng^- or ap^- clones are transformed into wing margin (Irvine and Wieschaus 1994; Williams et al. 1994). The N receptor is required for signaling in both directions, because loss of N function on one side of the D/V boundary eliminates wg expression and causes loss of wing tissue on both sides of the boundary (de Celis and Garcia-Bellido 1994; Rulifson and Blair 1995).

We propose that Dl acts as a ventral to dorsal signal that activates N to induce wing margin-specific genes during second-instar development. Dl displays three characteristics expected of such a signal: [1] Dl is re-
quired in ventral cells at the D/V boundary for wing margin formation; {2) DI is expressed at the D/V compartment boundary in second-instar discs; {3) ectopic DI can induce ectopic wing margin formation and wing outgrowth, but only in the dorsal compartment. For the DI signal, restriction of the response plays an important role in compartment specificity. For Ser, restriction of the signal, restriction of the response plays an important role in compartment cells. In turn, DI in ventral compartment cells signals back to dorsal compartment cells via N to induce wg and to reinforce DI expression or activity in ventral compartment cells. In turn, DI in ventral compartment cells signals back to dorsal compartment cells via N to induce wg and to reinforce DI expression or activity. Preliminary results indicate that ectopic DI expression induces Ser in the dorsal compartment of ptcG-4-DI discs (C. Micchelli, pers. comm.) and that ectopic Ser induces DI in the ventral compartment of ptcG-4-Ser discs (D. Doherty and G. Feger, unpubl.). Kim et al. (1995) have shown that fng acts upstream of Ser to activate Ser expression. The role of fng could be to initiate the positive feedback loop between DI and Ser either by signaling from dorsal to ventral cells to activate DI in the ventral cells, or by inhibiting the response to Ser and activating the response to DI in dorsal cells creating a border for DI and Ser signaling, or both. As wing development proceeds, the early pattern of DI expression evolves into the third-instar pattern where DI is expressed in two stripes flanking the wg-expressing cells at the D/V boundary. It is likely that the combined activities of wg, DI, Ser, and other genes generate the later expression pattern. This system enables an intrinsically asymmetric boundary between ap-expressing and -nonexpressing cells to induce the symmetric patterns of growth and gene expression required to form the wing.

**Conservation of mechanisms for axis formation and distal outgrowth in appendage development**

How axes are specified is a universal problem during appendage development. Data from a variety of species suggest that there may be a limited number of molecular mechanisms for generating axes and other pattern information. For example, the hedgehog pathway is used to pattern different types of appendages in animals as evolutionarily divergent as the fly, chicken, and mouse (for review, see Perrimon 1995; Tabin 1995). The DI-N signaling pathway described in this paper may also be used in vertebrate limbs. Jagged, a murine member of the DI/Ser family, is expressed in developing limbs (Lindsell et al. 1995). Wnt-7a, a mouse homolog of wg, has been shown to function in D/V patterning of limbs (Farr and McMahon 1995; Riddle et al. 1995). It remains to be determined whether the DI-N/Ser-N signaling pathways are used for limb axis formation throughout the animal kingdom.

**Materials and methods**

**Clonal analysis**

Adult mosaic clones of mutant DI tissue were generated by X-irradiating second-instar larvae, as described by Diaz-Benjumea and Cohen (1993). To mark the DI" chromosome, we used a forked" duplication on the third chromosome (distal to DI at cytological map position 9B), kindly provided by F. Diaz-Benjumea and S. Cohen, European Molecular Biology Laboratory [EMBL], Heidelberg, Germany. This allowed us to identify DI forked clones in flies hemizygous for pF6. We used Dper°, a loss-of-function DI allele (Heitzeiter and Simpson 1991), which gave no detectable DI protein in homozygous Dper° embryos.

For analysis in wing discs, mitotic clones lacking DI were generated using flipase-mediated mitotic recombination (Golic and Lindquist 1989; Golic 1991). We recombined Dper° and ebony, a recessive mutation that darkens cuticular structures, onto a chromosome carrying p[FRT, neo, ry+] at 82B (Xu and Rubin 1993). We crossed this chromosome into flies with a heat shock Myc-marked p[FRT, neo, ry+] third chromosome and a heat shock flipase X chromosome and generated homozygous clones in wing discs and adult wings (see Xu and Rubin 1993). We identified DI mutant clones by the absence of Myc expression in wing disc cells. Many wing discs with clones induced during second instar were extremely distorted, making them difficult to analyze.

**Immunocytochemistry**

Larvae were dissected in phosphate-buffered saline (PBS), fixed for 10 min in 4% formaldehyde and PEMS [0.1 M PIPES at pH 6.9, 1 mM EGTA, 2 mM MgSO4], rinsed several times in PBT, blocked for 1 hr at room temperature with 2% normal goat serum, and incubated overnight at 4°C with the primary antibody. After several washes with PBT, fluorescent-labeled secondary antibodies [Jackson Laboratories, USA] were added for 1–2 hr at room temperature, washed with PBT, and mounted in
glycerol/PBS/2% n-propylgallate. Samples were examined using a Bio-Rad MRC-600 confocal microscope. The following antibodies were used: rabbit anti-β-galactosidase [Cappel, USA] mouse anti-DI mAb 202 [Kooh et al. 1993], rabbit anti-Vg [Williams et al. 1991], rat anti-cut [Blanchinger et al. 1988], rabbit anti-N [E. Giniger and Y.N. Jan, in prep.], rabbit anti-wg [van den Heuvel et al. 1989], mouse anti-en [ Patel et al. 1989], rabbit anti-cubitus-interruptus [Schwartz et al. 1996], guinea pig anti-myc [C. Feger and Y.N. Jan, unpubl.]. Confocal figures were assembled using Photoshop 3.0 [Adobe, USA] and Canvas 3.5.3 [Deneba, USA].

Ectopic expression of N and D1

Targeted ectopic expression of N and D1 was accomplished using the GAL4 system [Brand and Perrimon 1993]. UAS–DI transgenic lines were generated by subcloning D1 cDNA 3.2 [Vaessin et al. 1987] into the pUAST vector and transformation into w– flies by standard techniques. UAS-activated N lines were generated by subcloning the transmembrane and intracellular domain sequences of N fused to the DI signal sequence into pUAST [E. Giniger, pers. comm.]. To test for wild-type function genie lines were generated by subcloning the GAL4 system [Brand and Perrimon 1993]. UAS-D1 trans- 
tibodies were used: rabbit anti-[B-galactosidase ICappel, USA)

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Acknowledgments

We thank S. Barbel and L. Ackerman for artwork, C. Bargmann, P. Kolodziej, and I. Clark for helpful comments on the manuscript, and S. Rafls for technical help with the GAL4 screen and the UAS nuclear lacZ construct. We thank J. Kim for helpful comments and for sharing his manuscript prior to publication. We are particularly indebted to E. Rulifson, C. Micchelli, and S. Blair for sharing results prior to publication, for helpful discussions, and for valuable comments on the manuscript. We would also like to thank T. Parody and M.A.T. Muskavitch for DI antibodies, R. Nusse for wg antibody, J. Gates and S.B. Carroll for vg antibody, C. Schwartz and T. Kornberg for en and ci antibody, E. Giniger for UAS– DI UAS–actN, and N antibody, L. Seugnet, M. Haenlin, and P. Simpson for making UAS–N available prior to publication as well as for D1rev10. B. Wilder for sharing results prior to publication, and F. Diaz-Benjumea and S. Cohen for the bld cu f+986 chromosome and sharing their manuscript prior to publication. This work was supported by the Howard Hughes Medical Institute. L.Y.J. and Y.N.J. are Howard Hughes investigators.

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*Genes Dev.* 1996, **10:**
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