Refinement of wingless Expression by a Wingless- and Notch-Responsive Homeodomain Protein, Defective Proventriculus

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Pattern formation during animal development is often induced by extracellular signaling molecules, known as morphogens, which are secreted from localized sources. During wing development in Drosophila, Wingless (Wg) is activated by Notch signaling along the dorsal–ventral boundary of the wing imaginal disc and acts as a morphogen to organize gene expression and cell growth. Expression of wg is restricted to a narrow stripe by Wg itself, repressing its own expression in adjacent cells. This refinement of wg expression is essential for specification of the wing margin. Here, we show that a homeodomain protein, Defective proventriculus (Dve), mediates the refinement of wg expression in both the wing disc and embryonic proventriculus, where dve expression requires Wg signaling. Our results provide evidence for a feedback mechanism that establishes the wg-expressing domain through the action of a Wg-induced gene product. © 2002 Elsevier Science (USA)

Key Words: Drosophila wing imaginal disc; Notch; wingless; defective proventriculus; morphogen; pattern formation.

INTRODUCTION

The body pattern of a multicellular organism forms as cells are proliferating. The spatial pattern of gene expression and cell growth are, therefore, coordinately regulated during development. In Drosophila, secreted proteins of Wingless (Wg) and Decapentaplegic (Dpp) act as key signaling molecules that organize gene expression and pattern formation in various tissues. In wing imaginal discs, Wg and Dpp are expressed at the dorsal–ventral (D-V) and anterior–posterior (A-P) compartment boundaries, respectively, and act as morphogens that confer positional information depending on their concentration (Lawrence and Struhl, 1996; Nellen et al., 1996; Neumann and Cohen, 1997; Zecca et al., 1996).

Wg expression in wing discs requires Notch (N) signaling along the D-V boundary. At the second larval instar, the dorsal region of wing imaginal discs is specified through the expression of a LIM-homeodomain protein, Apterous, and a glycosyltransferase, Fringe (Blair et al., 1994; Bruckner et al., 2000; Diaz-Benjumea and Cohen, 1993; Irvine and Wieschaus, 1994; Moloney et al., 2000; Williams et al., 1993). Apterous-expressing cells also express an N ligand, Serrate (Ser), which activates the N signal from the dorsal-to-ventral direction at the D-V boundary (Couso et al., 1995; de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995). Another N ligand, Delta (Dl), activates the N signal from the ventral-to-dorsal direction (Doherty et al., 1996).
The positive feedback loop between Ser and DI defines their expression at the D-V boundary until mid-third instar (de Celis and Bray, 1997; Panin et al., 1997). Upon N signal activation, several genes, such as wg and cut, are up-regulated at the D-V boundary to specify the wing margin (Irvine and Vogt, 1997; Neumann and Cohen, 1996; Rulifson and Blair, 1995). The expression of wg is refined by the Wg signal itself. Mosaic clones of dishevelled (dsh), a component of the Wg signal transduction pathway, lead to the expansion of Wg expression and result in the loss or duplication of the wing margin structure (Rulifson et al., 1996). Thus, the refinement of Wg expression is critical as to specification of the wing margin.

Cell growth is strictly coordinated with patterning of gene expression during imaginal disc development. The Wg function is essential for development and patterning of the wing; however, Wg alone cannot induce disc outgrowth in the wing pouch. The N signal plays a pivotal role in cell growth together with Wg and Vestigial (Vg) (de Celis and Bray, 1997; Go et al., 1998; Kim et al., 1996; Klein and Martinez-Arias, 1998). Therefore, the relationship between N signaling and the refinement of Wg expression appears to be important for the coordination of growth and patterning. Several lines of evidence suggest that the Wg signal input antagonizes the N signal through the direct interaction of Dsh and N (Axelrod et al., 1996) and that of Wg and N (Brennan et al., 1999a,b; Lawrence et al., 2001; Wesley, 1999). Wg-dependent expression of N ligands, DI and Ser, along the D-V boundary is also thought to antagonize N activity in a dominant-negative manner (de Celis and Bray, 1997; Klein et al., 1997; Michelli et al., 1997).

Here, we provide evidence that refinement of Wg expression requires a homeodomain protein, Defective proventriculus (Dve), which is responsive to both Wg and N signaling. The dve gene is required for embryonic development of the gut primordia, the proventriculus, and middle midgut, where it is activated by the Wg signal and Dpp signal, respectively (Fuss and Hoch, 1998; Nakagoshi et al., 1998). In the proventriculus, the expression of wg and dve initially overlaps, and then segregates into adjacent but exclusive domains (Nakagoshi et al., 1998). We show the expression pattern of dve in wing discs, which is triggered by the combined activities of Wg and Dpp and repressed along the D-V boundary by N-mediated signaling including the Wg and Dpp signals. We provide evidence that a Wg-target gene, dve, is required for the refinement of Wg expression in two distinct regions, the wing disc and embryonic proventriculus. These results suggest a feedback mechanism by which a morphogen-induced gene product refines the source of morphogen.

**MATERIALS AND METHODS**

**Fly Stocks**

The following Gal4/UAS strains were used: dpp-Gal4 (blk-Gal4-4A.3) (Morimura et al., 1996), vg-Gal4-181 (kindly provided by S. Morimura and F. M. Hoffmann), 30A-Gal4, UAS-IacZ (Brand and Perrimon, 1993), ptc-Gal4 (Hinz et al., 1994), AyGal4.25-UAS-GFP.S65T (Ito et al., 1997), UAS-dTCFβ13ΔN4 (Cadigan et al., 1998), UAS-tkvβ13Δ (Nellen et al., 1996), UAS-fluarm (Zecce et al., 1996), UAS-Ser (Spicher et al., 1994), UAS-Dl, UAS-Nact (Doherty et al., 1996), UAS-dad (Taneizumi et al., 1997), UAS-DERβ1 (O’Keefe et al., 1997), and UAS-dve-982 (Nakagoshi et al., 1998).

**Immunohistochemistry**

Mouse monoclonal antibodies to β-galactosidase (Gal) (Promega; 1:100 dilution), Myc (9E10; Santa Cruz Biotechnology; 1:50), and Cut (kindly provided by K. Kimura; 1:20), as well as rabbit anti-serum to Dve (1:100), and Ser (kindly provided by E. Knust and T. Murata; 1:5) were used together with Cy3- or Cy5-conjugated secondary antibodies (Jackson; 1:100) or with fluorescein isothiocyanate-conjugated antibodies to mouse immunoglobulin G (Cappel; 1:100). Peroxidase and immunofluorescence staining were performed as described (Nakagoshi et al., 1998). Confocal images were obtained with a Bio-Rad MRC-1024 or LEICA LSM TCS-NT.

**Mosaic Analyses**

Mosaic clones were generated by using the FRT- and FLP-mediated recombination system (Xu and Rubin, 1993). To generate tkv mosaic clones, y w; FRT40A/S6 ea flies were crossed with w HS-Flp; arm-lacZ FRT40A ones (Lecuit and Cohen, 1997) and the offspring were subjected to heat shock at 37°C for 10 min at the late first to early second larval instar.

To generate arm, dsh, N or zw3 mosaic clones, y w arm-FRT18A/FM7, y w dsh-FRT18A/FM7, N54321/FRT18A/FM7, or w zw3-FRT18A/FM7 females were crossed with W1296 FRT18A; hs-flp Sb/TM6 males (Rulifson and Blair, 1995), y w dsh1/FRT101/FM7 females were crossed with marm-FRT101; hs-FLP3 Sb/TM6 Tb (Rulifson et al., 1996), followed by heat shock as above. After 60–72 h (mid- to late third instar), imaginal discs were fixed and stained with anti-β-Gal monoclonal antibodies and anti-Dve serum.

To generate dve mosaic clones, we used FRT42-dve1 and y; FRT42-y; hs-flp flies for analysis of the adult wing phenotype and FRT42-dve1 and wg-lacZ FRT42-ma/Cyo; hs-flp flies for imaginal disc analyses. Mosaic clones expressing Nact were induced by crossing AyGal4.25-UAS-GFP.S65T and wg-lacZ UAS-Nact/Cyo; hs-flp flies and subjecting the offspring to heat shock at 37°C for 20 min at the second larval instar.

**RESULTS**

**Refinement of Wg Expression in the Embryonic Proventriculus**

The dve gene is essential for proventriculus development and is initially triggered by the Wg signal in its primordium. The expression of wg and dve initially overlaps, but then segregates into adjacent but exclusive domains (Nakagoshi et al., 1998). We examined the function of dve in wg expression. In the absence of dve, wg expression expands posteriorly to the region that normally expresses dve (Figs. 1A and 1B). This indicates that dve defines the posterior border of wg expression by repressing its expression. Thus,
Wg refines its expression domain via dve gene activation in the embryonic proventriculus.

**dve Expression in Wing Imaginal Discs**

Wg refines its own expression along the D-V boundary in wing imaginal discs (Rulifson et al., 1996). The above result of the dve function in the embryonic proventriculus led us to examine the possibility that dve acts in the refinement of wg expression at the D-V boundary. We first examined the expression of dve in wing discs.

At early to mid-third larval instar (48–24 h before pupariation, BP), dve expression begins throughout the prospective wing pouch, which overlaps partly with wg expression (Fig. 2A). The coexpression of dve and wg is obvious until mid-third instar, and subsequently, dve is excluded from the D-V boundary at mid- to late third instar (24–12 h BP; Fig. 2B). At this stage, dve is expressed complementary to wg. The activation of wg and repression of dve are both mediated by Notch (N) signaling at the D-V boundary (see below). At late third instar (12–0 h BP), dve expression is reduced in the distal region, whereas it remains strong at the proximal region of the wing pouch (Fig. 2C). The significance of this repression is unclear.

The embryonic expression of dve depends on the Wg signal in the proventriculus and on the Dpp signal in the middle midgut (Nakagoshi et al., 1998). We examined whether or not dve expression in wing discs depends on these signals. When the Wg signal was ectopically activated in a ring pattern around a wing pouch under the control of the 30A-Gal4 driver, dve was ectopically activated only in cells at the intersection of the ring with the A-P boundary, which normally expressed Dpp (Figs. 2D and 2E). In contrast, activation of the Dpp signal in the same ring pattern resulted in the ectopic expression of dve only at the D-V boundary, where Wg is normally expressed (Fig. 2F). Thus, the combined Wg and Dpp signals appear to induce dve expression in wing discs. To examine this possibility, we generated flip-out recombination clones that simultaneously express an activated form of the Dpp receptor (Tkv^{Q253D}) and that of a Wg signaling molecule (DArm). In these clones, some induced ectopic Dve expression autonomously outside the compartment boundaries (Figs. 2G–2I). These results strongly support the above notion that the combined activities of Wg and Dpp signals induce dve expression rather than other signals generated at compartment boundaries.

We next examined Dve expression in mosaic clones that failed to transduce the Wg or Dpp signal. When mutant clones of a thick veins allele, tkv^{a12}, were created in wing discs, large mutant clones were rarely observed despite the presence of wild-type clones of twin spots as previously reported (Nellen et al., 1996). In these discs, small mutant clones still expressed dve when they were generated within the Dve-expressing region (Figs. 3A–3C). Similar results were obtained on mosaic analyses of arm^{XM19}, dsh^{VA153}, and dsh^{v26} (Figs. 3D–3H; and data not shown). These observations suggest that Dve-expressing cells no longer need Wg and Dpp signals to maintain its expression. Alternatively, perdurance of signaling molecules in mutant clones might be enough to maintain dve expression. Taken together, we infer that the combined Wg and Dpp signals trigger dve gene expression. On the other hand, when these signals were inhibited in the region where dve was barely expressed around the D-V boundary, dve expression was rather enhanced to such levels as observed at the proximal region of the disc. Thus, Wg and Dpp signals in turn appear to be necessary for dve repression along the D-V boundary at mid- to late third instar (Figs. 3C and 3F).

**N Signaling Represses dve Expression along the D-V Boundary**

The expression pattern of dve raises the possibility that repression of dve along the D-V boundary is mediated by N
signaling, which plays a pivotal role in the activation of margin-patterning genes, such as \textit{wg} and \textit{cut}. To examine this possibility, the N signal was ectopically activated along the A-P boundary by expressing N ligands, Delta (Dl) and Serrate (Ser), under the control of patched (ptc)-Gal4 (Hinz et al., 1994). Dl and Ser trigger N signaling in the dorsal half and the ventral half of wing discs, respectively (Couso et al., 1995; de Celis et al., 1996; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996). In ptc-Gal4/UAS-Ser discs, N signaling is ectopically activated along the ventral A-P boundary and results in the ventral expression of the margin-patterning genes \textit{wg} and \textit{cut} (Diaz-Benjumea and Cohen, 1995; Kim et al., 1996; Fig. 4A). In these discs, dve expression was found to be repressed along the ventral A-P boundary (Figs. 4B and 4C). In ptc-Gal4/UAS-Dl discs, N signaling is ectopically activated along the dorsal A-P boundary, leading to dorsal dve suppression (Fig. 4D). Thus, the ectopic N signal can repress dve expression. In order to

\begin{figure}
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\caption{Dve expression in wing discs. (A–C) Dve (green), \textit{wg}-lacZ (red in A, B), and \textit{Cut} expression (red in C) at the early to mid- (A), mid- to late (B), and late third instar (C). (D–F) Ectopic activation of the Wg (D, E) or Dpp signal (F) in a ring pattern around the wing pouch (lacZ expression; brown in D). (D) 30A-Gal4 UAS-lacZ/UAS-flu-\textit{arm}. (E) 30A-Gal4/UAS-flu-\textit{arm}. (F) 30A-Gal4/UAS-tkv\textsuperscript{Q253D}. Arrows indicate ectopic Dve expression (black). (G–I) Simultaneous activation of Wg and Dpp signaling in flip-out clones (\textit{y w hs-flp; UAS-flu-\textit{arm} A\textit{yGal4.25-UAS-GFP.S65T}; UAS-tkv\textsuperscript{Q253D}/+) was marked by the expression of GFP (green in G). Dve expression (red in H) was ectopically induced outside the compartment boundaries (arrows). The merged image was shown in (I). Anterior is to the left, and dorsal is to the bottom in all discs in the following figures.}
\end{figure}
determine whether or not N indeed suppresses Dve expression along the D-V boundary of wing discs, we generated mosaic clones lacking N activity. The loss of N activity resulted in the ectopic activation of Dve in the region where Dve expression is normally suppressed along the D-V boundary (Figs. 4E–4G). These results indicate that N signaling represses dve along the D-V boundary of wing discs to create a domain in which Dve is absent and wg is activated.

As described above, Wg signaling appears to be necessary for dve repression along the D-V boundary. The action of Wg, which is up-regulated by N at the D-V boundary, might explain N-mediated repression of dve. To examine the cell autonomy for N-mediated repression of dve, we examined dve expression in N mutant clones at later stages. N mutant clones crossing the D-V boundary caused the derepression of Dve (Figs. 4H and 4I), with varying levels of dve expression within clones (Fig. 4K, arrows). This suggests that there is some nonautonomous effect on dve repression. We generated mutant mosaic clones for zeste-white 3 (zw3), in which Wg signaling is constitutively active. Partial repression of Dve was observed in zw3 mutant clones at early to mid-third instar (Figs. 5A–5C). At later stages, when N signaling is strongly activated along the D-V boundary, ectopic Dve repression in zw3 mutant clones was more evident outside the D-V boundary (Figs. 5D–5F). N-mediated dve repression thus depends largely on Wg signaling that is activated by N. At the late third instar, expansion of Dve repression at the distal region also depended on Wg and Dpp signaling. Inhibition of these signals by expressing a dominant-negative form of Wg signaling molecule (dTCFDN) or a negative regulator for Dpp signaling (dad) along the A-P boundary resulted in elevated expression of Dve (Figs. 5G and 5H). These results support the notion that N-mediated repression of dve requires Wg signaling.

**FIG. 3.** Dve expression in the absence of Dpp or Wg signaling. Mosaic clones of tkv	extsuperscript{122} (A–C), armXM19 (D–F), and dshVA153 (G, H) were marked by the absence of anti-β-Gal staining (green in A, D, G). Dve expression is shown in red (B, C, E, F, H). White arrows indicate the positions of mutant clones, and yellow arrows indicate the clones that were generated at the D-V boundary (A, C, D, F). (I) A late third wing disc of vg-Gal4/UAS-dTCFDN. The yellow arrow indicates ectopic dve expression along the D-V boundary.
repression of Dve has a nonautonomous effect, although Wg signaling alone is insufficient for complete repression at early stage. We infer that N-mediated events along the D-V boundary modulate the Wg and Dpp signaling, or another secreted signaling molecule, such as Spitz, might be involved in dve repression because the Spitz ligand is up-regulated along the D-V boundary (Nagaraj et al., 1999). Indeed, inhibition of EGF signaling by expressing a dominant-negative form of Drosophila EGF receptor (DERDN) along the A-P boundary resulted in derepression of Dve (Fig. 5I).

**Mutant Phenotypes of dve Mosaic Clones**

If Dve is important for the refinement of Wg expression at the D-V boundary of wing discs, dve activity must repress Wg expression outside the normal Wg stripe. To test this hypothesis, we generated mosaic clones in which dve expression was disrupted in wing discs. Expanded expression of Wg was apparent in dve mutant clones that intersected with the normal Wg stripe (Figs. 6A and 6B); the ectopic expression of Wg only occurred in mosaic clones immediately adjacent to the normal Wg stripe. How does Dve refine the Wg expression? Is it a downstream component of Wg signaling? To clarify the molecular mechanism of Wg refinement, we have examined the expression of Ser, which is a ligand for N and is up-regulated in a Wg-dependent manner. Accumulation of Ser and Dl besides the D-V boundary antagonizes N activity in a dominant-negative manner and is important for the refinement of N signaling along the D-V boundary (de Celis and Bray, 1997; Klein et al., 1997; Micchelli et al., 1997). If Dve is involved

**FIG. 4.** N signaling represses dve expression. (A–D) Ectopic activation of the N signal was induced along the A-P boundary. (A–C) ptc-Gal4/UAS-Ser discs. Ectopic expression of cut-lacZ (Jack et al., 1991) was observed in ventral region (red in A), where dve expression (green in B) was also repressed (arrows). (D) A ptc-Gal4/UAS-DI disc. Dve expression was repressed in dorsal region (arrows). (E–K) Dve expression (red) in Nf;mutant clones at mid- to late (E–G) and late third instar (H and K), respectively. Mosaic clones were marked by the absence of β-Gal staining (green). Merged images are shown in (G) and (I). Boxed region in (I) was magnified in (J) and (K). Clone boundaries were outlined in (I) and (K). Derepression of Dve was observed in mosaic clones crossing the D-V boundary (arrows in F–I), but was weak in some cells (arrows in K).
in these processes as a downstream component of Wg signaling. Ser expression would be lost in dve mutant clones. However, the expression of Ser was unaffected in dve mutant clones (Figs. 6C–6E), suggesting that Dve function is independent of these processes.

In adults, the generation of dve mutant clones resulted in duplication of margin bristles and wing margin notches (Figs. 6F–6I). Similar phenotypes have been observed for dsh mosaic clones (Axelrod et al., 1996; Couso et al., 1994; Figs. 6j and 6k) in which Wg expression is expanded (Rulifson et al., 1996). Although the mechanism producing notches is unclear, the expansion of Wg expression might be unable to specify the margin structure. These observations indicate that Dve represses Wg expression outside the normal Wg stripe.

**Ectopic Expression of dve along the D-V Boundary**

Given that Dve represses Wg expression, the absence of dve expression along the D-V boundary might be important for the expression of margin-patterning genes, including Wg. To test this possibility, we examined the effect of ectopic dve expression along the D-V boundary under the control of vestigial (vg)-Gal4. Such ectopic activation of dve resulted in a marked decrease of Wg expression in the anterior and posterior margin, but not in the distal margin (Figs. 7C and 7D). Similar pattern of Wg reduction is reported for discs mutant for spade, a regulatory mutation of Wg that reduces Wg function in the wing margin and in the wing hinge (Neumann and Cohen, 1996). Most strikingly, the expression of cut, which is activated by N along the D-V boundary (Micchelli et al., 1997), was completely suppressed by Dve (Figs. 7E and 7F). This ectopic activation of dve led to variable wing phenotype; some adults only had rudimentary wings (Fig. 7A) and others had notched wings (Figs. 7B). These defects observed in adult wings might be in part attributable to reduced expression of Wg and cut; however, it has not been reported that reduced expression of these genes at D-V boundary causes rudimentary wings.

**FIG. 5.** N-mediated Wg signaling is critical for dve repression. (A–F) Dve expression (red) in zw3 mutant clones at early to mid- (A–C) and mid- to late third instar (D–F), respectively. Mosaic clones were marked by the absence of β-Gal staining (green). Arrows indicate mosaic clones that the dve repression is partial (B) and almost complete (E). (G–I) Dve expression at late third instar by inhibiting various signals along the A-P boundary. (G) ptc-Gal4/UAS-dTCF ΔN. (H) dpp-Gal4/UAS-dad. (I) dpp-Gal4/UAS-DERΔN. Arrows indicate the derepressed Dve expression.
FIG. 6. Mutant phenotypes of dve mosaic clones. (A–E) *wg-lacZ* (A, B) and Ser (C–E) expression (red) in dve<sup>38</sup> mutant clones of wing discs. Mosaic clones were marked by the absence of anti-Myc staining (green). Arrows indicate mosaic clones in which *wg-lacZ* expression is expanded (B) or Ser expression is present (E). (F–I) dve<sup>1</sup> mosaic wings. (F, G) A dve<sup>1</sup> mosaic wing is shown in different focal planes. Mosaic clones of dve<sup>1</sup> are marked by yellow (y) bristles (arrowheads). Ectopic margin bristles derived from mutant clones (arrowheads) and wild-type cells (arrows) are indicated. Others exhibit similar ectopic bristles (H) and notched wings (I). (J, K) dsh<sup>75</sup> mosaic wings exhibit more severe phenotype for ectopic bristles (J) and notched wings (K).
Those defects might be rather due to a reduction of N activity as described below. Our results thus suggest that N-mediated repression of dve along the D-V boundary is crucial for the outgrowth and patterning of wing discs.

### Notch Signaling on dve and wg Expression

Mutant phenotypes of dve mosaic clones suggest the possibility that Dve refines the wg expression downstream of N signaling (Figs. 6C–6F). To investigate the relationship between N signaling and the Dve function in the regulation of wg expression, we examined the effect of Dve on wg activation mediated by N. Expression of a constitutively active form of N (Nact) along the A-P boundary resulted in ectopic wg expression (Fig. 7G). The simultaneous expression of both Nact and dve resulted in a decrease in this ectopic wg expression (Fig. 7H), supporting the above notion that Dve functions downstream of N in the regulation of wg expression.

We further examined the role of N in the expression of wg, cut, and dve by generating Nact-expressing clones. Flip-out recombination clones expressing Gal4 in larvae harboring UAS-Nact exhibit variable levels of Nact expression (de Celis and Bray, 1997). We detected two types of Nact clones in such wing discs (Fig. 8). In one type of clone, wg and cut were expressed and dve was repressed, similar to the situation at the D-V boundary (Figs. 8A and 8B; white arrows). This type of Nact clone reorganizes cell growth and gene expression to form ectopic wing pouches (de Celis and Bray, 1997). These clones also induced nonautonomous dve induction in adjacent cells (Figs. 8B, 8D, and 8F; yellow arrows). Such dve induction might reflect the generation of...
an ectopic wing pouch, namely ectopic outgrowth. In contrast, the second type of Nact clone did not exhibit wg activation or dve repression within clones and did not induce dve expression around them. wg- and cut-expressing clones appear to arise frequently in the region where dve expression is low; near the D-V boundary and outside the wing pouch. This observation is consistent with our finding that Dve represses N-mediated expression of wg and cut (Figs. 7C–7H). In addition, we often observed larger clones that did not express wg and cut in these regions (Figs. 8C–8F, arrowheads), indicating that the formation of wg- and cut-expressing clones does not necessarily depend on the clone location or size. Instead, it possibly depends on the strength of N signaling, which is reflected by the level of Gal4 expression, as visualized as green fluorescent protein (GFP) signals. Although most clones exhibited satu-

FIG. 8. Effects of Nact-expressing clones. Ectopic N signal activation was induced by the AyGal4 system, and Nact-expressing clones were marked by GFP expression (blue). (A–D) Mid to late third instar. (E, F) Late third instar. Dve expression is shown in green. The expression of N-target genes, Cut (A, D) and wg-lacZ (E), is shown in red. Some clones induced N-target gene expression (white arrows), and repressed dve expression within the wing pouch (B). Around these clones, ectopic Dve expression (green) was induced (yellow arrows in B, D, F). Note the prominent disc outgrowth at the late third instar (F). There was another type of clones that induced neither Cut, wg-lacZ nor ectopic Dve expression (arrowheads).
rated levels of the GFP signals, which make it difficult to
detect differences in GFP signal intensities, we were often
able to observe the two types of Nact-expressing clones
in which the intensity of GFP signals presumably reflected the
level of N signaling. A typical example is shown in Fig. 8. In
case of these, the first type of clones appear to express Nact at
higher levels (Fig. 8C, arrow) and the second type of clones
at lower levels of Nact (Fig. 8C, arrowhead). Altogether, we
suggest that the two types of Nact clones arise from the
difference in the level of N signaling within clones.

DISCUSSION

Roles of Wg, Dpp, and N Signaling
on dve Expression

Our study suggests that the combined activities of Wg
and Dpp induce the initial dve gene expression in wing
discs. However, the continuous input of these signals might
be unnecessary for its maintenance, although we could not
exclude the possibility that the perduration of signaling
molecules in tkv, arm, or dsh mutant clones was enough to
maintain Dve expression (Figs. 3A–3H). Interestingly, these
clones exhibited a rather high level of dve expression when
they were made at the D-V boundary (Figs. 3C and 3F).
Expression of a dominant-negative form of dTCF along the
D-V boundary also elevated dve expression (Fig. 3I). The
adults of such animals had notched wings resembling those
caused by ectopic dve expression along the D-V boundary (Fig. 7B).
Similar wing phenotypes have been observed by
inhibiting the Dpp signal along the D-V boundary (Tsuneizu
et al., 1997). These results suggest that Wg and Dpp signals
cause repression of dve at the D-V boundary after the
initial induction.

Such dual roles of Wg and Dpp signals in the expression of
a single gene have been reported for dachshund (dac)
expression in leg discs (Lecuit and Cohen, 1997). Initial
expression of dac is induced by Dpp and Wg signals,
whereas its maintenance is independent of these signals.
Furthermore, Dac expression is actively repressed by the
same signals at the center of the disc. Thus, the temporal
modes of action of Dpp and Wg are quite similar for
regulation of both the dac and dve genes. It remains unclear
how Dpp and Wg alter their actions toward the dac and dve
genes from positive to negative regulation.

Negative Feedback Mechanism to Refine
the Morphogen Source

In both wing discs and the proventriculus, the initially
overlapped expression of wg and dve becomes segregated
into complementary patterns via the ability of Dve to
suppress wg gene expression, which leads to refinement of
the border of the wg-expressing domain. In wing discs,
several different mechanisms limit wg expression to the
D-V boundary: (1) restriction of N activation to the D-V
boundary (Irvine and Vogt, 1997), which requires the Fringe
function, and a positive feedback loop between Ser and Dl
dexpression (de Celis and Bray, 1997; Irvine and Wieschaus,
1994; Klein and Martinez-Arias, 1998; Panin et al., 1997); (2)
the inhibition of N signaling in Ser- and Dl-expressing cells
in a dominant-negative manner (de Celis and Bray, 1997;
Klein et al., 1997; Michell et al., 1997); (3) the local
suppression by the Wg signal itself near the D-V boundary
(Rulifson et al., 1996). Our study has revealed a feedback
mechanism by which a Wg-induced gene product refines
the source of wg expression to shape a morphogen gradient.

Rulifson et al. (1996) showed that refinement of wg
expression is important to specify the structure of the wing
margin. In dsh mutant clones that abut the D-V boundary,
wg expression expands (Rulifson et al., 1996). The dve
mutant clone encompassing the D-V boundary also allows
the expansion of wg expression, as observed in dsh mutant
clones (Figs. 6A and 6B). The action of Dve in the refine-
ment of wg expression appears to attenuate N-mediated
gene expression. How is the Dve function related to the Wg
signaling cascade in this process? When dsh mutant clones
were created so as to abut the D-V boundary, dve expression
was still observed in such clones (Figs. 3G and 3H). These
observations for dsh clones suggest that Dve activity in the
absence of the Wg signal input is insufficient to refine wg
expression. On the other hand, when dve mutant clones
were created adjacent to the D-V boundary, wg expression
was expanded within the clones (Figs. 6A and 6B), but
Wg-dependent accumulation of Ser seemed to be normal
(Figs. 6C–6E). Thus, the Dsh-mediated Wg signal also
appears to be insufficient to refine wg expression in the
absence of Dve. Taken together, both Wg signaling and Dve
appear to be necessary for the refinement of wg. There
might be some interaction between Dve and Wg signaling
downstream of N.

The interaction with other transcription factors is also
important to clarify the molecular mechanism of wg refine-
ment. For instance, it is reported that POU domain pro-
teins, Nubbin and Drifter, are expressed in the wing pouch
(Certel et al., 2000; Cifuentes and Garcia-Bellido, 1997;
Neumann and Cohen, 1998). Especially, the expression of
drifter (also referred to as ventral veinless) is repressed
along the D-V boundary through Wg signaling, and mutant
clones induce ectopic bristles which have typical features of
margin bristles (Certel et al., 2000; de Celis et al., 1995).
The Drosophila homologue of mammalian serum response
factor (SRF), blistered, is also expressed in a similar pattern
to those of Dve and Drifter (Roch et al., 1998). Thus, they
might function in very similar ways or in the same pathway
to refine wg expression.

N-Mediated Signaling Coordinates Growth
and Patterning

N-mediated activation of wg together with Vg function
is important for disc growth (de Celis and Bray, 1997; Go
et al., 1998; Kim et al., 1996; Klein and Martinez-Arias, 1998).
In addition, repression of dve at the D-V boundary largely
depends on N-mediated Wg signaling and is also crucial for disc outgrowth and patterning of wing discs (Figs. 7A and 7B). Complementary pattern of dve and wg expression at mid- to late third instar appears to be important for wing patterning. How do these events organize growth and patterning? Our studies involving flip-out Nct clones might provide an insight in this issue (Fig. 8). These experiments suggested that the two types of Nct clones arise from a difference in the level of N signaling within clones: lower N signaling-clones express dve but not wg and cut, and higher N signaling-clones express wg and cut but not dve (Fig. 8). The second type of clone appears to mimic the situation at the D-V boundary. It is remarkable that the on and off states of dve expression within the clones were tightly correlated with the induction of N-target gene expression. Considering the ability of Dve to repress wg, this observation makes it possible to hypothesize a threshold of N-mediated signaling that defines both wg activation and subsequent dve repression; N-mediated signaling over this threshold can repress dve and results in the sustained expression of wg. Thus, it establishes a complementary pattern of dve and wg expression at the D-V boundary of wing discs at mid- to late third instar (Fig. 2B). This threshold also appears to define nonautonomous induction of cell growth (Fig. 8F). By utilizing the cold-sensitivity of Gal4 to drive gene expression, we were able to induce different levels of N signaling. These experiments also suggested the notion that the level of N signaling that represses dve is important for disc outgrowth (data not shown). Thus, our model assuming a threshold of the N-mediated signal repressing dve might provide a clue for understanding the coordination between cell growth and patterning through shaping of the Wg stripe.

ACKNOWLEDGMENTS

We thank S. Morimura, F. M. Hoffmann, K. Kimura, T. Tabata, K. Ito, T. Murata, S. Hayashi, K. Basler, S. S. Blair, S. M. Cohen, M. Haenlin, R. Nusse, and the Bloomington Stock Center for the fly strains; K. Kimura for the anti-Cut antibodies, E. Knust and T. Hama for the use of the confocal microscope. This work was partly supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture (to H.N.); and by grants from PRESTO (to H.N.) and CREST (to F.M.) of Japan Science and Technology Corporation.

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Received for publication April 8, 2002
Revised May 29, 2002
Accepted May 30, 2002
Published online August 7, 2002