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Direct and Long-Range Action of a Wingless Morphogen Gradient

Myriam Zecca,* Konrad Basler,* and Gary Struhl†
*Zoologisches Institut der Universität Zürich
Winterthurerstrasse 190
8057 Zürich
Switzerland
†Howard Hughes Medical Institute
Columbia University College of Physicians and Surgeons
New York, New York 10032

Summary

Wingless (Wg), a founding member of the Wingless/Int-1 (Wnt) family of secreted proteins, acts as a short-range inducer and as a long-range organizer during Drosophila development. Here, we determine the consequences of ectopically expressing (i) a wild-type form of Wg, (ii) a membrane-tethered form of Wg, and (iii) a constitutively active form of the cytosolic protein Armadillo (Arm), which normally acts to transduce Wg, and we compare them with the effects of removing endogenous Wg or Arm activity. Our results indicate that wild-type Wg acts at long range, up-regulating the transcription of particular target genes as a function of concentration and distance from secreting cells. In contrast, tethered Wg and Arm have only short-range or autonomous effects, respectively, on the transcription of these genes. We interpret these findings as evidence that Wg can act directly and at long range as a gradient morphogen during normal development.

Introduction

It has long been appreciated that the organization of cell and body patterns might be controlled by gradients of "form-producing" substances or morphogens (Morgan, 1897; Turing, 1952; reviewed in Slack, 1987). However, the long-range organizing effects attributed to such gradients could also be achieved by inducers that act at short range and in sequence to confer spatial information across a tissue (Spemann, 1938). During the past 10 years, secreted proteins of the Wnt, Hedgehog, and Transforming Growth Factor-β families have been shown to have long-range organizing activities during tissue development (reviewed in Roelink, 1995). The identification of such organizing molecules now allows us to ask whether their long-range effects are mediated by gradient or sequential inductive mechanisms.

The protein encoded by the Drosophila gene *wingless* (*wg*) is a defining member of the Wnt family (reviewed in Nusse and Varmus, 1992). In embryos, Wg acts as a short-range inducer: it is secreted by subpopulations of cells within each segment and received by their immediate, nonsecreting neighbors (van-den-Heuvel et al., 1989), which respond by expressing particular target genes (DiNardo et al., 1988; Martinez-Arias et al., 1988; Vincent and Lawrence, 1994). However, Wg also appears to organize the pattern of cells located at a distance from secreting cells, during embryogenesis (Nüsslein-Volhard and Wieschaus, 1980; Bejsovec and Martinez-Arias, 1991; Hoppler and Bienz, 1995) and during the development of adult tissues (Struhl and Basler, 1993; Diaz-Benjumea and Cohen, 1995). Moreover, the strength of this organizing influence appears to depend on the amount of Wg secreted (Struhl and Basler, 1993). Such evidence has led to the proposal that Wg acts in some contexts as a gradient morphogen (Bejsovec and Martinez-Arias, 1991; Struhl and Basler, 1993; Hoppler and Bienz, 1995). At present, this hypothesis remains unproven and controversial because of the failure to distinguish it from models in which Wg acts by proxy as a short-range inducer (e.g., see Diaz-Benjumea and Cohen, 1994; Vincent, 1994).

Here, we have sought to resolve this controversy by performing the following experiments. First, we have compared the effects of ectopically expressing (i) a wild-type form of Wg, (ii) a membrane-tethered form of Wg, or (iii) a constitutively active form of the protein Armadillo (Arm), which normally acts within cells to transduce Wg. If Wg exerts its long-range action through a mechanism of sequential induction, then the ectopic expression of tethered Wg or of constitutively active Arm in a defined subpopulation of cells should exert similar long-range consequences to that of "free" Wg. This is because all three conditions should induce any downstream signals and propagate the inductive cascade. In contrast, if Wg acts directly and at long range as a gradient morphogen, tethering Wg to the cell surface should limit its effective range, and constitutively activating Arm should cause a cell-autonomous response. Second, we have tested whether Wg-signal transduction is required continuously in cells whose behavior is controlled by Wg-secreting cells located at a distance. A sequential-inductive model would predict no, whereas a gradient model would predict yes. Finally, we have assayed whether Wg-secreting cells can elicit distinct outputs in surrounding cells as a function of the concentration of Wg protein secreted, as would be expected for a gradient mechanism but not for a sequential-inductive mechanism. We have performed these experiments in cells giving rise to the wings, legs, and eyes. We find that, in all cases examined, our results argue against a sequential-inductive model of Wg function and in favor of the proposal that Wg functions as a morphogen.

Results

Target Genes for Wg Signaling in the Developing Wing

The Drosophila wing derives from two populations of cells, the anterior and posterior compartments, which arise side by side during embryogenesis and are further subdivided into dorsal and ventral subcompartments during larval life (Bryant, 1970; Garcia-Bellido et al., 1973). Once allocated, cells of the dorsal and ventral compartments interact across the compartment boundary to induce the expression of *wg* in a narrow stripe of

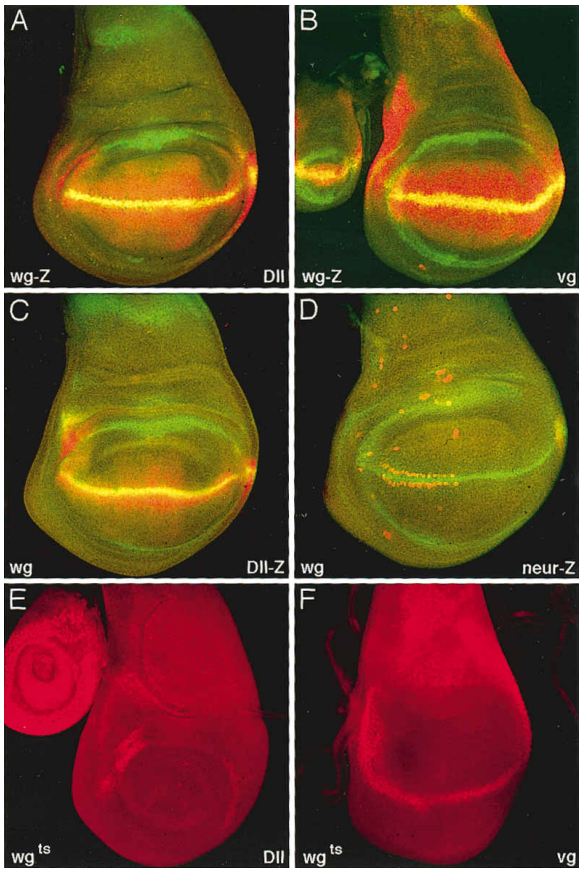

Figure 1. Target Genes for Wg Signaling in the Developing Wing

(A–D) *Dll* (A), *vg* (B), *Dll-lacZ* (C), and *neur-lacZ* (D) expression (red) relative to *wg-lacZ* (A and B) or *wg* (C and D) expression (green) in the wing disc (as monitored by the expression of their protein products). *wg*-expressing cells along the D/V compartment boundary appear yellow: note that the stripes of *Dll*, *vg*, and *Dll-lacZ* expression straddle the stripe of *wg* expression, and that *Dll-lacZ* expression is significantly narrower than that of the *Dll* and *vg* expression.

(E and F) *Dll* (E) and *vg* (F) expression in wg^{ts} wing discs 2 days after a shift to nonpermissive temperature: *Dll* expression in the wing is abolished, and *vg* expression is reduced to a thin stripe of cells along the D/V compartment boundary (see text). At least 20 wg^{ts} mutant discs were analyzed, all of which showed loss of *Dll* and *vg* expression. Here and in all remaining images of wing discs, dorsal is at the top and anterior is to the left.

cells that straddles the boundary (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995). This stripe marks the future margin of the wing and is associated with overlying stripes of expression of the genes *Distalless* (*Dll*) and *vestigial* (*vg*), which extend many cell diameters on either side of the *wg*-expressing cells (Figures 1A and 1B) (Williams et al., 1991; Carroll et al., 1994) and encode putative transcription factors required for normal wing development (Cohen et al., 1989; Williams et al., 1991; Kim et al., 1996; G. Campbell, personal communication). The boundaries of expression of *Dll* and *vg*, as visualized by the expression of Dll and Vg protein, are not sharp; instead, expression grades out over a few cell diameters at the edges of each stripe (Figures 1A and 1B). *wg*-expressing cells along the dorsoventral (D/V) compartment boundary are also associated with the segregation of rows of neuroblasts on either side: these neuroblasts can be visualized by the expression of a *lacZ* reporter allele of the gene *neuralized* (*neur-lacZ*) and give rise to the characteristic rows of bristles that decorate the wing margin (Figure 1D) (Boulianne et al., 1991; Blair, 1993; Phillips and Whittle, 1993; Couso et al., 1994).

Two lines of evidence suggest that *Dll* and *vg* expression as well as bristle specification are organized by Wg. First, using a temperature-sensitive mutation of *wg*, it is possible to remove *wg* activity at chosen times during wing development. As shown in Figures 1E and 1F, *Dll* expression is abolished within 48 hr following a shift to the nonpermissive temperature, and *vg* expression is eliminated except for a thin stripe of cells straddling the D/V compartment boundary (these cells express *vg* principally in response to signaling by Delta and Serrate: Kim et al., 1996, and citations therein). Similarly, previous studies have shown that late loss of *wg* activity prevents the specification of wing-margin bristles (Blair, 1993; Phillips and Whittle, 1993; Couso et al., 1994). Second, ectopic expression of *wg* (see below; Diaz-Benjumea and Cohen, 1995), as well as ectopic activation of the Wg-signal transduction pathway, caused by eliminating the Zeste-white 3/Shaggy kinase (Blair, 1992; Diaz-Benjumea and Cohen, 1995), up-regulates the expression of *Dll* and *vg* within the wing-blade primordium and causes ectopic expression of *neur-lacZ*. Thus, within this tissue, the normal patterns of *Dll* and *vg* expression, as well as of *neur-lacZ* expression and bristle differentiation, appear to be governed by Wg.

Tethered Wg

Attempts to generate forms of Wg, which are tethered to the membrane by being fused at the C-terminus to single-pass transmembrane proteins, have generally yielded proteins that have little or no detectable biological activity (Parkin et al., 1993; data not shown). Consequently, we constructed a gene, *Nrt-flu-wg*, in which Wg is fused at its N-terminus to the C-terminus of Drosophila Neurotactin (Nrt), a type-II transmembrane protein (Hortsch et al., 1990), with three copies of the Flu epitope tag inserted at the site of fusion (see Experimental Procedures). As a control, we also generated a *flu-wg* gene, which encodes a protein in which three Flu tags are inserted at the same position in the N-terminus of Wg (Experimental Procedures).

To compare the activity of the Wg, Flu-Wg, and Nrt-Flu-Wg proteins, we expressed each of these proteins indiscriminately during embryogenesis using either the constitutive *Tubulinα1* promoter (Experimental Procedures) (Basler and Struhl, 1994; Zecca et al., 1995) or the UAS/Gal4 technique (Fischer et al., 1988; Brand and Perrimon, 1993). In all three cases, such embryos show similar "naked" phenotypes in which the prominent belts of ventral denticles normally positioned in the anterior portion of each segment are replaced partially or completely by naked cuticle (data not shown). As previously described (Noordermeer et al., 1992), the suppression of ventral denticles serves as an assay for ectopic Wg signaling, and by this criterion, Nrt-Flu-Wg, like Flu-Wg and Wg, has activity. We have also compared the ability of Flu-Wg and Nrt-Flu-Wg to overcome the absence of

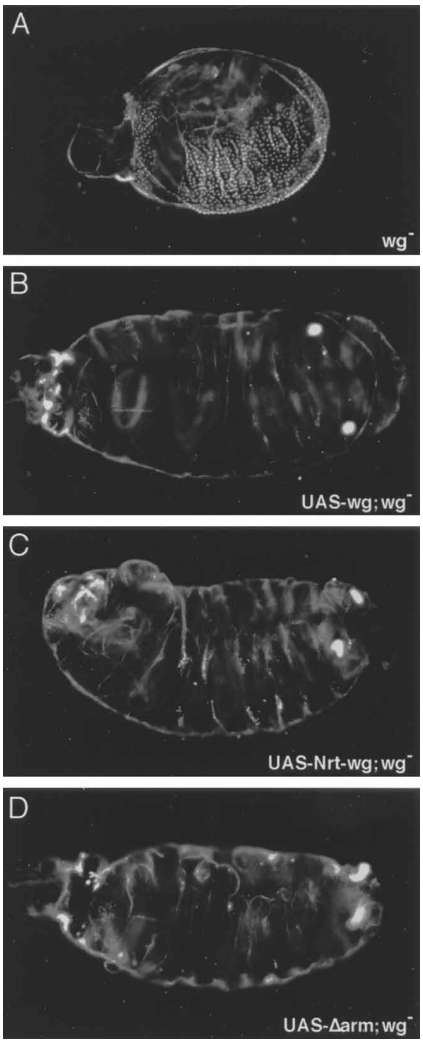

Figure 2. Activation of the Wg-Signal Transduction Pathway by Ubiquitous Expression of Free Wg, Tethered Wg, and Activated Arm Proteins in *wg*⁻ Embryos

(A) *wg*⁻ embryos give rise to abnormally short larvae, which form a "lawn" of ventral denticles.

(B–D) Larvae derived from *UAS-flu-wg; wg*⁻ (B), *UAS-Nrt-flu-wg; wg*⁻ (C), and *UAS-flu-Δarm; wg*⁻ (D) embryos in which the UAS-transgenes were ubiquitously expressed under the control of a *hs-Gal4* driver gene. In all three cases, the lawn phenotype is reversed, and the larvae show the characteristic "naked" phenotype associated with constitutive Wg signaling.

endogenous *wg* activity by repeating the experiment in *wg*⁻ embryos (see Experimental Procedures). Embryos lacking endogenous *wg* function give rise to larvae in which all ventral epidermal cells form a "lawn" of denticles (Figure 2A) (Nüsslein-Volhard and Wieschaus, 1980): ubiquitous expression of either *UAS-flu-wg* or *UAS-Nrt-flu-wg* can reverse this phenotype, generating larvae that show a naked phenotype (Figures 2B and 2C).

Constitutively Active Arm

The Arm protein acts autonomously within cells to transduce the Wg signal (Wieschaus and Riggleman, 1987; Peifer et al., 1991). Because (i) Wg signal-transducing activity appears to map to the C-terminal portion of the protein (Peifer and Wieschaus, 1990), and (ii), Wg-signal transduction is correlated with dephosphorylation of an N-terminal residue of the protein (Yost et al., 1996), we generated a Flu-tagged truncated form of the Arm gene, termed *flu-Δarm*, in which the coding sequence for two copies of the Flu tag replace the coding sequence for the N-terminal domain of the native protein (Experimental Procedures).

As described above for *flu-wg* and *Nrt-flu-wg*, we find that ubiquitous expression of *flu-Δarm* under UAS/Gal4 control causes naked phenotypes (data not shown). To determine whether this activity is ligand independent, we tested whether *UAS-flu-Δarm* expression can reverse the "lawn" phenotype of *wg*⁻ mutant embryos. As shown in Figure 2D, this is indeed the case.

Distinct Long-Range, Short-Range, and Cell-Autonomous Responses to Free Wg, Tethered Wg, and Constitutively Activated Arm

To assay the signaling capacities of Flu-Wg, Nrt-Flu-Wg, and Flu-ΔArm, we have generated clones of marked cells expressing these proteins and assayed the consequences on gene expression and on the final pattern differentiated in the adult. To do this, we have combined the Flp-out (Struhl and Basler, 1993) and Gal4/UAS (Fischer et al., 1988; Brand and Perrimon, 1993) techniques as recently described by Nellen et al. (1996) (see also Experimental Procedures). For example, to obtain clones of *flu-wg*-expressing cells, we generated larvae carrying the transgenes *UAS>CD2,y*⁺*>flu-wg* and *hs-flp*, as well as the Gal4 driver gene *C765*, which directs high levels of expression from the UAS promoter in wing disc cells (Nellen et al., 1996). Excision of the *>CD2,y*⁺*>* Flp-out cassette following a mild heat shock generates clones of *UAS>flu-wg*-expressing cells marked (i) by the loss of CD2 expression and the *y*⁺ gene and (ii) by the gain of Flu-Wg expression. *vg*, *Dll*, and *neur-lacZ* expression were assayed by the expression of their protein products, as in Figure 1. In addition, we monitored expression of a *Dll-lacZ* gene, which gives rise to a stripe of β-gal expression that is significantly narrower than that of endogenous Dll-protein expression and has steeply graded edges (Figure 1C).

Cells that express *flu-wg*, *Nrt-flu-wg*, or *flu-Δarm* invariably express high levels of *vg* and *Dll-lacZ*, provided that they are located within the wing-blade primordium where these genes are normally responsive to Wg (Figure 3; the expression of *Dll* in this experiment and subsequent experiments is similar to that of *vg* expression and is not shown). However, these cells differ dramatically in their ability to up-regulate *vg*, *Dll*, and *Dll-lacZ* expression in surrounding cells. *UAS>flu-wg* cells up-regulate *vg* and *Dll* expression in wild-type cells up to 10 or more cell diameters away (Figure 3A) and up-regulate *Dll-lacZ* expression in cells up to 5 or more cell diameters away (Figure 3D). In each case, the boundary of up-regulated expression is not sharp but declines in a graded fashion over a few cell diameters. In contrast, *UAS>Nrt-flu-wg* cells up-regulate the expression of all three genes only in their immediate wild-type neighbors, and in this case,

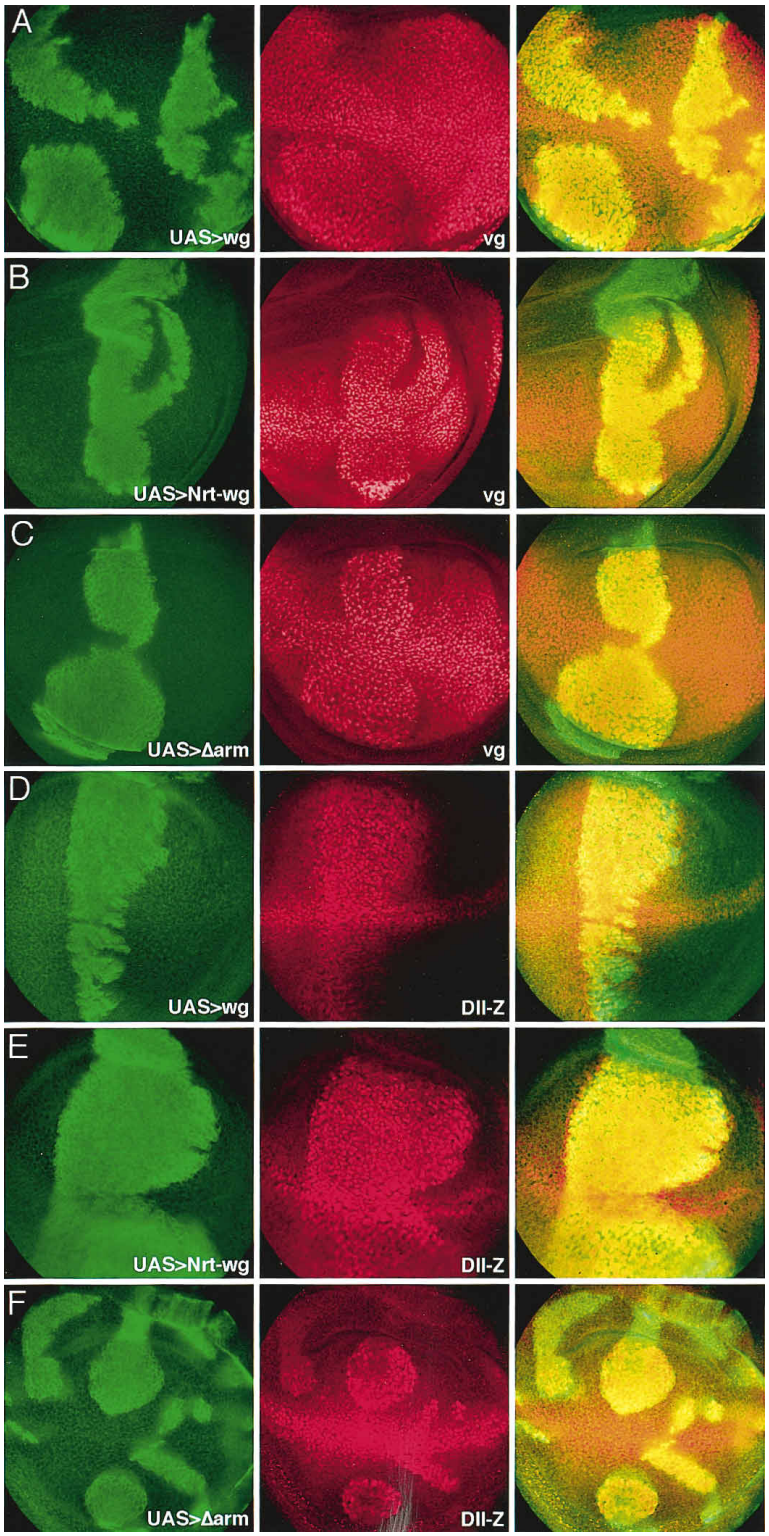

Figure 3. Long-Range, Short-Range, and Autonomous Responses to Free Wg, Tethered Wg, or Constitutively Activated Arm in the Developing Wing

(A–C) Ectopic *vg* expression (monitored by expression of Vg protein; red) associated with wing discs containing clones of *UAS>flu-wg* (A), *UAS>Nrt-flu-wg* (B), and *UAS>flu-Δarm* (C) cells (monitored by expression of the Flu epitope; green).

(D–F) Ectopic *Dll-lacZ* expression (red) associated with wing discs containing clones of *UAS>flu-wg* (D), *UAS>Nrt-flu-wg* (E), and *UAS>flu-Δarm* (F) cells (green). Note that clones of *UAS>flu-wg* cells up-regulate *vg* and *Dll-lacZ* expression in cells up to at least 10–15 cell diameters away, whereas clones of *UAS>Nrt-flu-wg* cells induce only their immediate, wild-type neighbors to express *vg* and *Dll-lacZ*, and *UAS>flu-Δarm* clones have a strictly cell-autonomous effect on both genes. Clones were induced by a single 30 min heat shock of 36°C during the first or second instar. In each experiment, at least 50 discs carrying one clone or more were analyzed, and all of the clones behaved similarly.

there is a sharp boundary of expression (Figures 3B and 3E). Finally, *UAS-flu-Δarm* cells do not up-regulate the expression of any of these genes in surrounding cells (Figures 3C and 3F).

Equivalent results were obtained when we assayed *neur-lacZ* expression in the disc or bristle differentiation in the adult wing. As shown in Figure 4, cells within *UAS>flu-wg*, *UAS>Nrt-flu-wg*, and *UAS>flu-Δarm* clones often express *neur-lacZ* ectopically and give rise to clusters of adventitious y^- bristles in the adult wing. However, only *UAS>flu-wg* and *UAS>Nrt-flu-wg* induce neighboring wild-type cells to express *neur-lacZ* and form

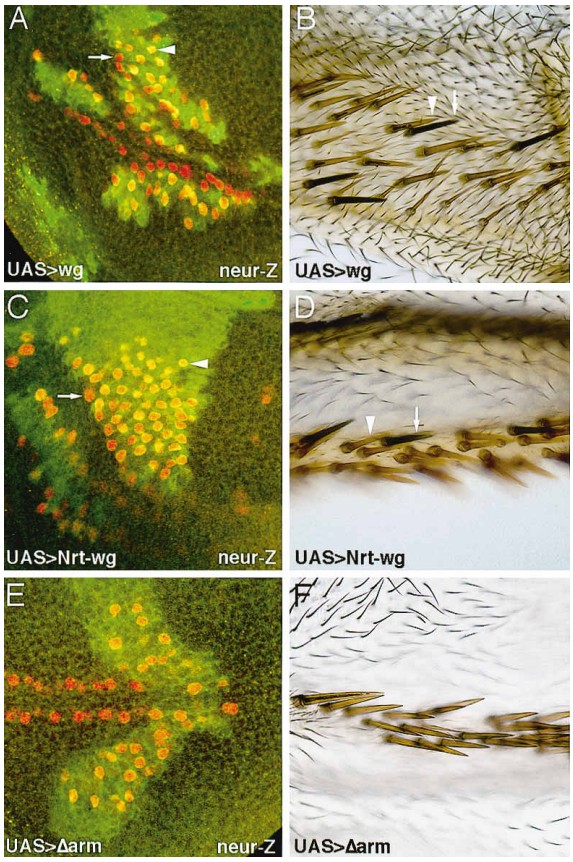

Figure 4. Induction of *neur-lacZ* Expression and Bristle Differentiation by Free Wg, Tethered Wg, and Activated Arm

(A, C, and E) Ectopic *neur-lacZ* expression (red) associated with wing discs containing clones of *UAS>flu-wg* (A), *UAS>Nrt-flu-wg* (C), and *UAS-flu-Δarm* (E) cells (monitored by expression of the Flu epitope; green). Note that both *UAS>flu-wg* and *UAS>Nrt-flu-wg* cells can induce neighboring, wild-type cells to express *neur-lacZ* (arrows), whereas *UAS>flu-Δarm* cells cannot (*neur-lacZ*-expressing cells within the clones are marked with arrowheads in (A) and (C). (B, D, and F) Clones of *UAS>flu-wg* (B) and *UAS>Nrt-flu-wg* (D) cells, marked by the *yellow* (*y*) mutation, can differentiate ectopic bristles (arrowheads) and induce surrounding, wild-type (y^+) *cells to form ectopic bristles (arrows). In contrast, clones of UAS>flu-Δarm* (F) cells differentiate ectopic bristles in a strictly autonomous fashion, as indicated by the absence of ectopic y^+ bristles. Clones were induced by a single 30 or 60 min heat shock of 33°C during the first or second instar. In each experiment, at least 20 clones were analyzed, all of which behaved similarly.

bristles (Figures 4A–4D), whereas *UAS>flu-Δarm* cells do not (Figure 4E and 4F). We note that *UAS>flu-wg* cells induce ectopic *neur-lacZ* expression only in surrounding wild-type cells in the immediate vicinity, indicating that *UAS>flu-wg* cells have a short-range nonautonomous influence on this output, in contrast to their long-range influence on *vg*, *Dll*, and *Dll-lacZ* expression (Figures 3A and 3D).

To examine the distribution of Flu-Wg and Nrt-Flu-Wg expressed by *UAS>flu-wg* and *UAS>Nrt-flu-wg* cells, we assayed the expression of these proteins using anti-Flu and anti-Wg antisera, counterstaining in the case of Wg antisera with antisera directed against the CD2 reporter protein, which marks all cells outside of the clone. We are unable to detect the Flu or Wg epitopes associated with wild-type cells surrounding the clones of misexpressing cells, even though staining for both epitopes is intense in cells belonging to these clones (e.g., Figure 4A, and data not shown). Hence, even though secreted Flu-Wg appears to act directly on wild-type cells several cell diameters away, the level of Flu-Wg to which they respond is sufficiently low to fall beneath our level of detection. Although *UAS>flu-wg* and *UAS>Nrt-flu-wg* cells could, in principle, cause nonautonomous effects on surrounding cells by expressing the endogenous *wg* gene, we find no evidence that such clones are associated with ectopic expression of a *lacZ*-expressing form of the endogenous *wg* gene in the wing primordium (data not shown).

In summary, *UAS>flu-wg* cells can exert a long-range and graded influence on the *vg*, *Dll*, and *Dll-lacZ* expression in surrounding wild-type cells. In contrast, *UAS>Nrt-flu-wg* cells exert only a short-range, all-or-none influence, and *UAS>flu-Δarm* cells have no effect on surrounding cells.

Arm Is Required Autonomously and Continuously to Mediate the Response of Wing Cells to Wg-Secreting Cells Located at a Distance

The results described above provide evidence that Wg acts directly, rather than by proxy, to elicit *Dll* and *vg* expression in nonsecreting cells. To test whether Wg signal transduction is required continuously to sustain *vg* and *Dll* expression in these cells, we have used the FLP/FRT mitotic-recombination technique (Golic, 1991) to reduce *arm* function in single cells and their descendents and noted whether there is a corresponding loss of *vg* and *Dll* expression (see Experimental Procedures).

In general, clones of *arm* mutant cells proliferate only for 24–36 hr (3–4 cell divisions) after they are generated in the presumptive wing blade; after this, the mutant cells stop dividing and either die or are actively eliminated from the disc epithelium (data not shown). When stained for either Vg or Dll expression 36 hr after mitotic recombination is induced, all of the cells within such clones fail to express either protein (Figures 5A and 5B). To control for the possibility that these cells are simply dead, we have also stained for expression the Engrailed protein, which is normally expressed in all cells of the posterior compartment (Hama et al., 1990) and is unstable (Heemskerk et al., 1991). We find that most, although not all, such clones express Engrailed (Figure 5C), indicating that their failure to express Vg or Dll is unlikely to be due to their being dead. We also note that *arm* mutant clones in other portions of the wing disc, such as the presumptive notum, proliferate normally. Because the proliferative behavior of *arm* mutant clones appears similar to that of *vg* mutant clones (Kim et al., 1996), it is possible that the former do not proliferate in the wing blade because they fail to express *vg*. Thus, we infer that wing cells positioned far from *wg*-expressing cells must receive direct input from Wg to sustain the expression of *vg* and *Dll*. This conclusion is also supported by our finding that late loss of endogenous wg^{ts} activity causes the loss of expression of both genes (Figures 1D and 1E) and provides an independent line of

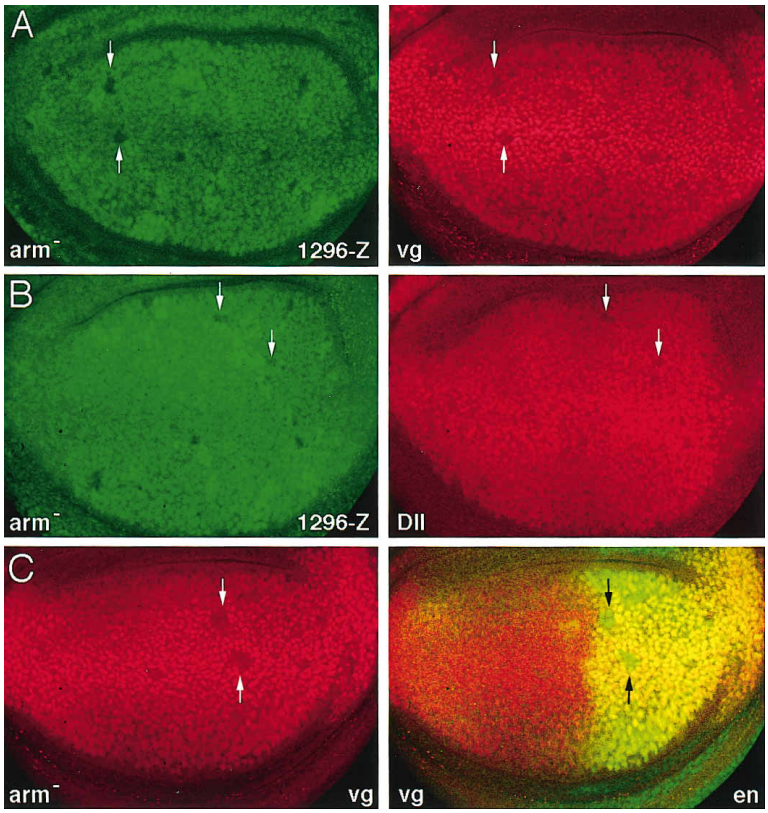

Figure 5. Arm Is Required Autonomously and Continuously to Maintain *vg* and *Dll* Expression in Response to Wg Signaling

(A–B) Clones of arm^{XM19} cells, marked by the absence of *WG1296-lacZ* expression (green), fail autonomously to express *vg* (A) or *Dll* (B), as monitored by the expression of Vg and Dll protein (red), even when located far from *wg*-expressing cells along the D/V compartment boundary.

(C) Most clones of arm^{XM19}cells obtained in the posterior compartment under the same conditions as in (A) and (B), and monitored by the loss of Vg expression (red), show normal levels of En expression (green), indicating that they are still alive. Arrows indicate examples of clones. In each experiment, at least 50 discs were analyzed, each carrying at least five clones.

evidence to that of the ectopic-expression experiments described above (Figures 3 and 4), indicating that secreted Wg acts directly and at long range on surrounding cells.

Distinct Responses Are Elicited at Different Distances from Wg-Secreting Cells and at Different Threshold Concentrations of Wg

Comparing the patterns of *neur-lacZ*, *Dll-lacZ*, *Dll*, and *vg* expression relative to endogenous *wg*-expressing cells or to clones of *UAS>flu-wg* cells, it appears that *wg*-expressing cells induce only their immediate neighbors to express *neur-lacZ*, whereas *wg*-expressing cells exert an intermediate-range influence on *Dll-lacZ* expression and a long-range influence on *Dll* and *vg* expression. Because our results indicate that the expression of these genes reflects the response of cells to the direct action of Wg, we suggest that Wg accumulates as a gradient in tissue surrounding *wg*-expressing cells and induces transcription of these genes when Wg exceeds distinct concentration thresholds. We have performed two additional tests of this hypothesis.

First, we have examined the relative domains of ectopic *neur-lacZ*, *Dll-lacZ*, and *vg* expression associated with clones of *UAS>flu-wg* cells by double-labeling experiments. As expected from the patterns of *vg* and *neur-lacZ* expression assayed relative to *UAS>flu-wg* cells (Figures 3A and 4A), we find that *vg* and *neur-lacZ* are expressed in nested circles; cells in the inner circle express both genes, and cells in the outer circle express only *vg* (Figure 6A). Similar results were obtained when we compared *Dll-lacZ* and *vg* expression, except that the nested circles of expression are closer in size, the boundary of *Dll-lacZ* expression that defines the inner circle coming within a few cell diameters of that of *vg* expression (Figure 6B). Thus, Wg appears to have the capacity to define at least three distinct outputs; moreover, these outputs are elicited over different distances from *UAS>flu-wg* cells, consistent with the view that secreted Flu-Wg accumulates as a gradient and induces each response when it exceeds a different threshold concentration.

Second, we have examined the consequences of expressing different concentrations of ectopic Wg on the transcription of *Dll-lacZ* and *neur-lacZ*. In this case, we have made use of a *Tubα1>CD2,y⁺>flu-wg* transgene in which the constitutive, relatively low level promoter from the *Tubulinα1* gene is used to drive expression of Flu-Wg. As shown in Figure 6C, we observe that most clones of *Tubα1>flu-wg* cells (14/19) are associated with elevated levels of *Dll-lacZ*; however, these clones are only rarely associated with ectopic *neur-lacZ*-expressing cells in the disc (1/32; Figure 6D). In this respect, they differ markedly from *UAS>flu-wg* clones, virtually all of which are associated with ectopic *neur-lacZ*-expressing cells (Figure 4A) as well as high levels of *Dll-lacZ* expression (Figure 3D). We have also compared Flu-Wg expression in *Tubα1>flu-wg* versus *UAS>flu-wg* cells and observed that the latter express levels of Flu-Wg that are at least 5– to 10-fold higher (data not shown). Hence, we can attribute the different extents of ectopic

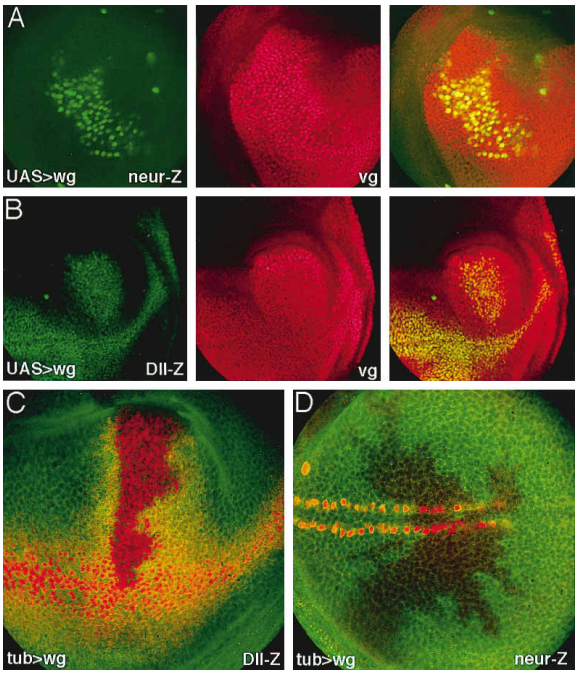

Figure 6. Distinct Responses Are Elicited at Different Distances from Wg-Secreting Cells and at Different Threshold Concentrations of Wg

(A) *neur-lacZ* (green) and *vg* (red) expression associated with a clone of *UAS>flu-wg* cells. Note that *neur-lacZ* and *vg* are expressed in nested circles, both genes being expressed in the inner circle and only *vg* being expressed in the outer circle.

(B) *Dll-lacZ* (green) and *vg* (red) expression associated with a clone of *UAS>flu-wg* cells. Note that *Dll-lacZ* and *vg* are expressed in nested circles, both genes being expressed in the inner circle and only *vg* being expressed in the outer circle. However, the inner circle of cells expressing both genes is much larger relative to the outer circle than when *neur-lacZ* and *vg* expression are monitored (A). For each experiment, 20 clones in the anterior wing primordium were analyzed, and all behaved similarly.

(C and D) *Dll-lacZ* (C; red) and *neur-lacZ* (D; red) expression in discs bearing clones of *Tubα1>wg* cells (monitored by the loss of CD2 expression, green). Note that *Dll-lacZ* expression is up-regulated throughout the clone shown in (C) as well as surrounding wild-type cells, whereas *neur-lacZ* is not ectopically expressed in cells in the clone shown in (D). Clones were induced as in Figure 3.

neur-lacZ expression associated with the two types of clones to differences in the level of Flu-Wg expression.

Evidence for Direct and Long-Range Action of Wg in the Developing Leg and Eye

In addition to its localized activity in the developing wing, *wg* is also expressed in tightly restricted patterns in other imaginal discs. In the leg, *wg* is expressed in a narrow, ventral wedge immediately anterior to the anteroposterior compartment boundary (Figure 7A) (Struhl and Basler, 1993 and citations therein), and this domain of expression falls within a much broader wedge of expression of the reporter gene *H15-lacZ* (Figure 7B) (Wilder and Perrimon, 1995). *wg* is also expressed in tightly restricted domains along the dorsal and ventral edges of the developing eye (Figure 7C) (Treisman and Rubin, 1995), a domain of expression that falls within the broader expression domains of the *optomotor-blind* (*omb*) gene (Figure 7D) (Grimm and Pflugfelder, 1996). We find that *UAS>flu-wg* cells can exert a long-range nonautonomous influence on *H15-lacZ* expression in the leg (Figure 7E) and on *omb-lacZ* expression in the eye (Figure 7H), in contrast to *UAS>Nrt-flu-wg* and *UAS>flu-Δarm* cells, which respectively exert short-range (Figures 7F and 7I) or strictly autonomous (Figures 7G and 7J) influences on the expression of these genes. Hence, we conclude that Wg can organize the expression of a number of genes in different contexts by acting directly and at long range.

Discussion

Wnt proteins are one of the first examples of secreted proteins that have been shown to have long-range organizing activities during animal development (McMahon and Moon, 1989; Struhl and Basler, 1993). As with members of the Hedgehog and Transforming Growth Factor-β families similarly associated with long-range organizing activities (reviewed in Roelink, 1995), speculation about their mode of action has raised a classic controversy extending back to the early history of embryology (Morgan, 1897; Spemann, 1938; reviewed in Slack, 1987). The gist of this controversy is that there are at least two ways in which such molecules can exert a long-range influence on cellular behavior: by acting directly and at long range on responding cells, or by acting indirectly and at short range through the induction of other signaling molecules. As recently shown for Hedgehog (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995) and the Transforming Growth Factor-β homolog Decapentaplegic (Dpp) (Lecuit et al., 1996; Nellen et al., 1996), these alternatives can be distinguished by comparing the consequences of ectopically expressing these proteins with the consequences of constitutively activating the systems that normally serve to receive and transduce them. For molecules functioning as short-range inducers, such as Hedgehog, both experimental interventions cause long-range reorganizations of growth and patterning because both cause the expression of secondary signaling molecules. In contrast, for molecules functioning as long-range morphogens, such as Dpp, only the ectopic expression of the ligand, and not the constitutive activation of its intracellular transduction system, has this property. Here, we extend this experimental paradigm to Wg and find evidence that it, like Dpp, has the expected properties of a gradient morphogen.

Direct and Long-Range Action of Wg

We have concentrated on the expression of a series of genes, *vg*, *Dll*, *H15*, and *omb*, which have the common property that they are normally expressed in broad domains in response to thin stripes of Wg-secreting cells. Our main finding is that these long-range outputs depend critically on the ability of Wg to move from expressing cells. The failure of either tethered Wg or constitutively activated Arm to exert an equivalent long-range influence to that of secreted Wg on the expression of these genes argues against the possibility that Wg activates their transcription by proxy through the induction

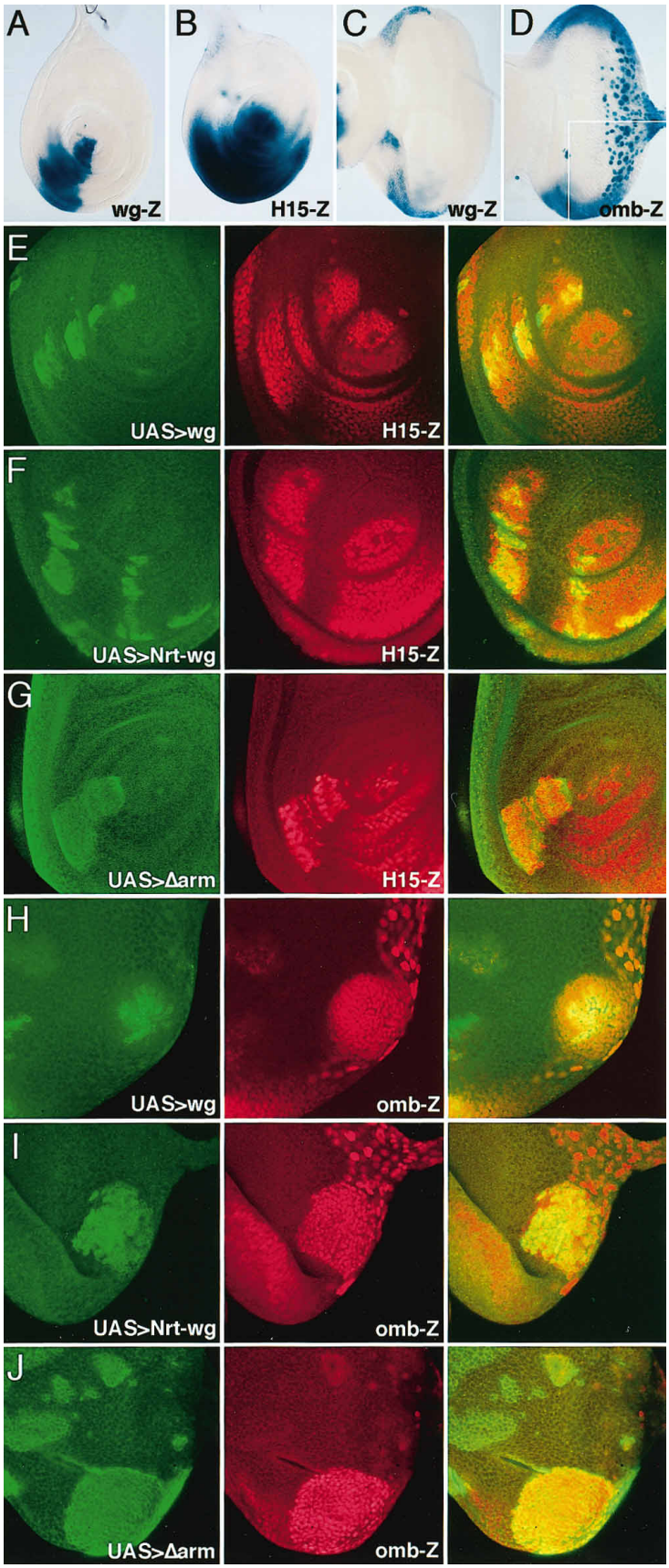

Figure 7. Long-Range, Short-Range, and Autonomous Responses to Free Wg, Tethered Wg, or Constitutively Activated Arm in the Developing Leg and Eye

(A–D) *wg-lacZ* (A) and *H15-lacZ* (B) expression in the leg imaginal disc and *wg-lacZ* (C) and *omb-lacZ* (D) expression in the eye disc. Note that the *H15-lacZ* and *omb-lacZ* domains of expression overlay and are broader than the domains of *wg-lacZ* expression in each disc (*omb-lacZ* is also expressed in a crescent of glial cells, which can be seen as large dots of blue staining [this expression is unrelated to Wg signaling]); the boxed region in (D) is shown at higher magnification in the images shown in (H), (I), and (J).

(E–G) Ectopic *H15-lacZ* expression (red) associated with clones of *UAS>flu-wg* (E), *UAS>Nrt-flu-wg* (F), and *UAS>flu-Δarm* (G) cells (monitored by expression of the Flu epitope; green).

(H–J) Ectopic *omb-lacZ* expression (red) associated with clones of *UAS>flu-wg* (H), *UAS>Nrt-flu-wg* (I), and *UAS>flu-Δarm* (J) cells (green); the *UAS>flu-wg* cells (H) are marked with a Wg antisera, whereas the *UAS>Nrt-flu-wg* (I) and *UAS>flu-Δarm* cells (J) are marked with the Flu antisera. Note that clones of *UAS>flu-wg* cells up-regulate *H15-lacZ* and *omb-lacZ* expression at long range relative to clones of *UAS>Nrt-flu-wg* cells, which induce only their immediate, wild-type neighbors to express these genes and relative to clones of *UAS>flu-Δarm* cells, which have a strictly cell-autonomous effect. Note also that the *UAS>flu-Δarm* clones in the middle of the eye disc shown in (J) do not express *omb-lacZ*, suggesting that these cells are not competent to respond to Wg by expressing *omb-lacZ*. Clones were induced as in Figure 3. In each experiment, at least 30 discs carrying one clone or more were analyzed, and all behaved similarly.

of other signaling molecules. This conclusion is reinforced by an independent line of evidence, which is our finding that Wg signaling is required autonomously and continuously in cells located at a distance from Wg-secreting cells to sustain the broad domains of expression of *Dll* and *vg*.

These results also argue against a "cellular-memory" mechanism such as that recently proposed to account for the long-range action of Dpp (Lecuit et al., 1996). According to this model, Wg would act directly but only at short range to alter surrounding cells so that they and their descendents would heritably express genes like *vg* and *Dll* even if they moved out of contact with Wg-secreting cells (e.g., as a consequence of cell proliferation). However, we observe that *vg* and *Dll* expression are eliminated in cells located at a distance from Wg-secreting cells by the late loss of Wg signaling (e.g., in clones of *arm* mutant cells), in direct conflict with such a cellular-memory model. In addition, if the memory model were correct, the expression of tethered Wg should suffice, like that of secreted Wg, to alter cellular memory and generate broad domains of *vg*, *Dll*, *H15*, and *omb* expression, which is not the case. We note that even for the case of Dpp signaling, the argument for a cellular-memory mechanism is undermined by the observation that the continuous expression of *omb* and *spalt*, two genes that respond over a long range to Dpp signaling in the wing, depends on the continuous activity of intracellular components required to transduce Dpp (Lecuit et al., 1996; Nellen et al., 1996).

If one accepts the argument that the expression of *vg*, *Dll*, *H15*, and *omb* depends on the direct and continuous input of Wg signaling, then their normal patterns of expression have several implications for the range and distribution of secreted Wg. First, these genes are normally expressed in cells up to at least 20 cell diameters away from Wg-secreting cells (e.g., as is clearly the case for *Dll* and *vg* expression in the wing disc; Figures 1A and 1B). Hence, we infer that the range of direct action of Wg may extend as much as 20 or more cell diameters away from *wg*-expressing cells. However, we cannot visualize Wg associated with non-*wg*-epressing cells even when these cells are located near or next to Wg-secreting cells, indicating that the secreted protein is present only at low concentration, beneath our level of detection. It is important to emphasize that we do not know how Wg moves across presumptive wing tissue. Studies of the evolving pattern of *vg* expression during wing development (Williams et al., 1993; Kim et al., 1996) suggest that the domain of *vg* broadens slowly and in a way that correlates with cell proliferation and hence challenges the simple notion that Wg diffuses freely from secreting cells. A similar situation is observed for the apparent movement of Dpp along the anteroposterior axis of the wing primordium, as inferred by its ability to induce *omb* expression (Nellen et al., 1996).

Second, all cells within the prospective wing blade appear to be equally responsive to Wg signaling in our ectopic-expression experiments. Moreover, *vg*, *Dll*, and *neur-lacZ* are expressed in broad stripes centered on the narrow stripe of *wg*-expressing cells, indicating an equal and symmetrical influence of Wg protein emanating from the vicinity of the D/V compartment boundary on the expression of these target genes. A curious exception to this general rule is the failure of *wg*-expressing cells to transcribe the *neur-lacZ* reporter gene or to form bristles (Blair, 1993; Phillips and Whittle, 1993; Couso et al., 1994) even though *wg*-expressing cells should be exposed to maximal levels of Wg protein. However, the inductive interactions between dorsal- and ventral-compartment cells that are responsible for driving *wg* expression in these cells are mediated by the Notch receptor (Kim et al., 1996, and citations therein), raising the possibility that Notch activity in these cells precludes their differentiating as bristles. Such interactions do not occur in cells located at a distance from the D/V boundary, and these cells respond to ectopic Wg, tethered Wg, and activated-Arm expression by transcribing *neur-lacZ* and differentiating as bristles.

Third, the boundaries of expression of *vg*, *Dll*, and *Dll-lacZ* are not sharp but rather decline in a graded fashion as a function of distance from Wg-secreting cells. This suggests that secreted Wg accumulates as a concentration gradient in tissue surrounding *wg*-expressing cells and that cells can respond in quantitatively, as well as qualitatively, distinct ways to this gradient. It is notable that *vg*, *Dll*, and *omb* encode putative transcription factors (the *H15* gene has not yet been characterized at the molecular level). Consequently, their graded expression in response to Wg suggests that a concentration gradient of extracellular Wg can establish one or more concentration gradients of transcription factors within tissues. This situation is reminiscent of the control of body patterning by the putative extracellular ligands Spätzle and Trunk in the early, syncytial embryo (Morisato and Anderson, 1994; Casanova et al., 1995). In all three cases, the local expression of a putative extracellular morphogen appears to be transduced directly into the graded distributions of transcription factors within cells, such as Vg, Dll, and Omb for Wg, Dorsal for Spätzle (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989), and Huckebein and Tailless for Trunk (Pignoni et al., 1990; Brönner and Jäckle, 1991).

There are at least two complicating factors to our interpretation of Wg signaling, particularly in the wing. First, *wg* is expressed not only along the D/V compartment boundary of the wing imaginal disc but also in other domains, particularly in two rings of cells surrounding the presumptive wing blade, which will form the proximal hinge portion of the adult wing (e.g., Figure 1) (Neumann and Cohen, 1996). However, the graded distributions of *vg*, *Dll*, and *Dll-lacZ* expression within the wing blade suggest that these cells are responding only to Wg emanating from the D/V compartment boundary. In support of this view, selective loss of *wg* activity in these presumptive hinge cells has little effect on growth or patterning within the wing blade (Neumann and Cohen, 1996). Thus, Wg secreted by cells in the prospective wing hinge may have only limited access to or influence on cells that will give rise to the wing blade.

The second complicating factor is that *vg* expression in the presumptive wing blade also appears to depend on Dpp emanating from anterior compartment cells along the anteroposterior compartment boundary (Kim et al., 1996). Hence, Dpp and Wg may act in combination to induce *vg* transcription, possibly by acting together

on the regulation of the recently identified "quadrant" enhancer in intron IV of the *vg* gene (Kim et al., 1996).

Distinct Outputs Elicited by Different Threshold Concentrations of Wg

In addition to its capacity to act at long range, another clear expectation of a gradient morphogen is that different threshold concentrations will elicit distinct outputs with "promorphological" value (Wilson, 1925), allowing the gradient to organize a cellular pattern. In the wing, we can define at least two outputs with different thresholds, namely the expression of *vg*, which normally directs the proliferation and differentiation of wing-blade cells (Kim et al., 1996), and the expression of the gene *neur*, which encodes a putative transcription factor required autonomously for bristle formation in adult tissues (Dietrich and Campos-Ortega, 1984; Boulianne et al., 1991). Further, we provide evidence that *neur* expression requires a relatively high concentration of Wg activity, whereas *vg* (and *Dll*) expression require much lower levels. As a consequence, Wg emanating from cells along the D/V compartment boundary appears to define the limits of a broad domain of wing tissue as well as a narrow stripe of bristle-forming cells that run along the wing margin.

In addition to these two outputs, we have also found that a *Dll-lacZ* reporter gene is expressed in intermediate domain with a boundary that falls between the boundaries of *vg* and *neur-lacZ* expression. Although we do not know whether this boundary has any relevance to the normal activity of the *Dll* gene, it nevertheless establishes that the putative gradient of Wg has the instructional capacity to define at least three thresholds, each eliciting a different response. It is notable that *Dll*-gene function is required for the normal differentiation of cells in the vicinity of the wing margin, including *neur-lacZ*-expressing cells, which would normally form the margin bristles (G. Campbell, personal communication). Hence, the induction of *Dll* expression by Wg, like that of *vg* and *neur-lacZ* expression, may also play a significant role in patterning the wing.

We note that the pathway leading from signaling by a putative morphogen, such as Wg, to the transcriptional regulation of subordinate genes and ultimately to the final cell pattern need not be direct (as discussed in Nellen et al., 1996; Lawrence and Struhl, 1996). This is particularly so for *neur-lacZ* expression, which is induced in isolated cells close to the D/V boundary rather than in a swath of cells, all of which appear to be responding in a uniform fashion to Wg. *neur-lacZ*-expressing cells are neuroblasts that arise from a population of proneural cells by a process of lateral specification (Rulifson and Blair, 1995). In this case, Wg appears to define the population of proneural cells, and these cells then send signals other than Wg, which are transduced by the Notch receptor, leading to the segregation of the *neur-lacZ*-expressing cells.

Morphogen Gradients

The existence of morphogen gradients in multicellular systems has long been a matter of conjecture (Morgan, 1897; Spemann, 1938; reviewed in Slack, 1987). In our opinion, the present results with Wg and recent results with Dpp (Lecuit et al., 1996; Nellen et al., 1996) provide a strong argument for accepting the existence of morphogen gradients as a fact and for focusing further analyses on how such gradients arise and how they organize growth and pattern.

Experimental Procedures

Transgenes

(i) *UAS>CD2,y⁺>flu-wg*, *UAS>CD2,y⁺>Nrt-flu-wg*, *UAS>CD2,y⁺>flu-Δarm*, and *Tub>CD2, y+>flu-wg* transgenes were generated as described previously (Basler and Struhl, 1994; Zecca et al., 1995; Nellen et al., 1996). The amino-acid sequences of the joins between the Flu, Wg, Nrt, and Arm peptides of the Flu-Wg, Nrt-Flu-Wg, and Flu-ΔArm are as follows: Flu-Wg: **R**^{32}AR[YPYDVPDYA]$_3$SS^{36}. . . (the Flu-epitope sequences are derived from the influenza virus hemagglutinin protein HA1 [Wilson et al., 1984] and are bracketed; the amino acids from Wg are shown in bold); Nrt-Flu-Wg: AR^{844}[YPYDVPDYA]$_3$SS^{36}. . . (amino acids from Nrt are shown in italics, and the Flu-Wg sequences appear as above); Flu-ΔArm: MGNKCCSKRQGTMA GNI[YPYDVPDYA]$_2$GSE^{155}. . . C^{843}GSPPKTTRKVED (the Flu sequences are bracketed; the amino acids from Arm are shown in bold; the Flu-ΔArm coding sequence is flanked at the N- and C-terminal ends by a myristalization signal and an inactive nuclear localization signal).

(ii) *hs-Gal4* and *C765-Gal4* driver genes (Brand and Perrimon, 1993; Nellen et al., 1996).

(iii) *neur-lacZ*, *wg-lacZ*, *H15-lacZ*, and *omb-lacZ* reporter genes (Boulianne et al., 1991; Phillips and Whittle, 1993; Wilder and Perrimon, 1995; Grimm and Pflugfelder, 1996; Nellen et al., 1996).

(iv) The *Dll-lacZ* gene is a P-element insertion allele of *Dll* (*Dll^{01092}*; Spradling et al., 1995) identified by M. Singer and W. Gelbart (personal communication).

Ectopic Expression

(i) The *UAS-flu-wg*, *UAS-Nrt-flu-wg*, and *UAS-flu-Δarm* genes were indiscriminately expressed in embryos by outcrossing males carrying these genes to females carrying an *hs-Gal4* driver line, and heat shocking the embryos thrice at 37°C for 30 min with a recovery time of 2 hr at 25°C between heat shocks. To perform this experiment in *wg*$^-$ embryos, *wg^{CX4}stc/CyO; hs-Gal4/+* females were crossed to *wg^{CX4}stc/+; UAS-flu-wg/+* males, and the progeny were subjected to the same heat-shock regime. The *stc* mutation (Jiang and Struhl, 1995) causes tufts of dorsal hairs to form in place of single hairs and allows *wg^{CX4}stc* homozygous larvae to be identified, regardless of whether they exhibit a "lawn" or "naked" phenotype. *wg^{CX4}* is a null allele of *wg*.

(ii) Generation and analysis of Flp-out clones was performed as described previously (Struhl and Basler, 1993; Basler and Struhl, 1994; Zecca et al., 1995; Nellen et al., 1996) using the *C765-Gal4* driver to direct constitutive expression, mediated by the UAS-enhancer element (see Brand and Perrimon, 1993) using the *neur-lacZ*, *Dll-lacZ*, *wg-lacZ*, *omb-lacZ*, and *H15-lacZ* reporter genes when appropriate. Heat-shock conditions used to generate clones in each experiment are noted in the Figure legends. Analyses were performed on imaginal discs and were removed from mid- to late-third instar larvae. Expression of proteins carrying a Flu-tag was monitored by using the monoclonal antibody 12CA5 (BAbCO).

(iii) *wg^{ts}* animals were maintained at permissive temperature of 16°C and shifted to a nonpermissive temperature of 29°C for 48 hr prior to analysis.

Clones Lacking *arm* Function

Clones of cells lacking *arm* function and marked by the loss of the *WG1296-lacZ* reporter gene, which expresses *lacZ* in all wing cells, were generated by Flp-mediated mitotic recombination (Golic, 1991; Xu and Rubin, 1993) by subjecting larvae of the genotype *y w arm^{XM19} FRT18/WG1296 FRT18; hs-flp* to a single 60 min heat shock of 37°C 36 hr prior to fixation at the end of the third larval instar.

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References

Basler, K., and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. Nature *368*, 208–214.

Bejsovec, A., and Martinez-Arias, A. (1991). Roles of wingless in patterning the larval epidermis of Drosophila. Development *113*, 471–485.

Blair, S. (1992). *shaggy* (*zeste-white 3*) and the formation of supernumerary bristle precursors in the developing wing blade of *Drosophila*. Dev. Biol. *152*, 263–278.

Blair, S.S. (1993). Mechanisms of compartment formation: evidence that non-proliferating cells do not play a critical role in defining the D/V lineage restriction in the developing wing of *Drosophila*. Development *119*, 339–351.

Boulianne, G.L., de la Concha, A., Campos-Ortega, J.A., Jan, L.Y., and Jan, Y.N. (1991). The *Drosophila* neurogenetic gene *neuralized* encodes a novel protein and is expressed in precursors of larval and adult neurons. EMBO J. *10*, 2975–2983.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Brönner, G., and Jäckle, H. (1991). Control and function of terminal gap gene activity in the posterior pole region of the *Drosophila* embryo. Mech. Dev. *35*, 205–211.

Bryant, P.J. (1970). Cell lineage relationships in the imaginal wing disc of Drosophila melanogaster. Dev. Biol. *22*, 389–411.

Carroll, S.B., Gates, J., Keys, D.N., Paddock, S.W., Panganiban, G. E.F., Selegue, J.E., and Williams, J.A. (1994). Pattern formation and eyespot determination in butterfly wings. Science *265*, 109–114.

Casanova, J., Furriols, M., McCormick, C.A., and Struhl, G. (1995). Similarities between trunk and spätzle, putative extracellular ligands specifying body pattern in *Drosophila*. Genes Dev. *9*, 2539–2544.

Cohen, S.M., Bronner, G., Kuttner, F., Jurgens, G., and Jackle, H. (1989). Distal-less encodes a homoeodomain protein required for limb development in Drosophila. Nature *338*, 432–434.

Couso, J.P., Bishop, S.A., and Martinez Arias, A. (1994). The wingless signalling pathway and the patterning of the wing margin in Drosophila. Development *120*, 621–636.

Diaz-Benjumea, F.J., and Cohen, S.M. (1994). *wingless* acts through the *shaggy/zeste-white 3* kinase to direct dorsal-ventral axis formation in the *Drosophila* leg. Development *120*, 1661–1668.

Diaz-Benjumea, F.J., and Cohen, S.M. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. Development *121*, 4215–4225.

Dietrich, U., and Campos-Ortega, J.A. (1984). The expression of neurogenic loci in imaginal epidermal cells of Drosophila melanogaster. J. Neurogenet. *1*, 315–332.

DiNardo, S., Sher, E., Heemskerk, J.J., Kassis, J.A., and O'Farrell, P.H. (1988). Two-tiered regulation of spatially patterned engrailed gene expression during Drosophila embryogenesis. Nature *332*, 604–609.

Fischer, J.A., Giniger, E., Maniatis, T., and Ptashne, M. (1988). GAL4 activates transcription in *Drosophila*. Nature *332*, 853–855.

Garcia-Bellido, A., Ripoll, P., and Morata, G. (1973). Developmental compartmentalisation of the wing disk of *Drosophila*. Nature New Biol. *245*, 251–253.

Golic, K. (1991). Site-specific recombination between homologous chromosomes in *Drosophila*. Science *252*, 958–961.

Grimm, S., and Pflugfelder, G.O. (1996). Control of the gene *optomotor-blind* in *Drosophila* wing development by *decapentaplegic* and *wingless*. Science *271*, 1601–1604.

Hama, C., Ali, Z., and Kornberg, T.B. (1990). Region-specific recombination and expression are directed by portions of the *Drosophila engrailed* promoter. Genes Dev. *4*, 1079–1093.

Heemskerk, J., DiNardo, S., Kostriken, R., and OFarrell, P.H. (1991). Multiple modes of engrailed regulation in the progression towards cell fate determination. Nature *352*, 404–410.

Hoppler, S., and Bienz, M. (1995). Two different thresholds of *wingless* signalling with distinct develomental consequences in the *Drosophila* midgut. EMBO J. *14*, 5016–5026.

Hortsch, M., Patel, N.H., Bieber, A.J., Traquina, Z.R., and Goodman, C.S. (1990). *Drosophila* neurotactin, a surface glycoprotein with homology to serine esterases, is dynamically expressed during embryogenesis. Development *110*, 1327–1340.

Jiang, J., and Struhl, G. (1995). Protein kinase A and Hedgehog signalling in *Drosophila* limb development. Cell *80*, 563–572.

Kim, J., Irvine, K.D., and Carroll, S. (1995). Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing Drosophila wing. Cell *82*, 795–802.

Kim, J., Sebring, A., Esch, J., Kraus, M.E., Vorwerk, K., Magee, J., and Carroll, S.B. (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila vestigial* gene. Nature *382*, 133–138.

Lecuit, T., Brook, W.J., Ng, M., Callega, M., Sun, H., and Cohen, S.M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. Nature *381*, 387–393.

Lepage, T., Cohen, S.M., Diaz-Benjumea, F.J., and Parkhurst, S.M. (1995). Signal transduction by cAMP-dependent protein kinase A in *Drosophila* limb patterning. Nature *373*, 711–715.

Li, W., Ohlmeyer, J.T., Lane, M.E., and Kalderon, D. (1995). Function of protein kinase A in hedghehog signal transduction and Drosophila imaginal disc development. Cell *80*, 553–562.

Martinez-Arias, A., Baker, N.E., and Ingham, P.W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the Drosophila embryo. Development *103*, 157–170.

McMahon, A.P., and Moon, R.T. (1989). int-1—a proto-oncogene involved in cell signalling. Development *107*, 161–167.

Morgan, T.H. (1897). Regeneration in *Allolobophora foetida*. Roux's Arch. Dev. Biol. *5*, 570–586.

Morisato, D., and Anderson, K.V. (1994). The *spätzle* gene encodes a component of the extracellular signaling pathway establishing the dorso-ventral pattern of the Drosophila embryo. Cell *76*, 677–688.

Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. Cell *85*, 357–368.

Neumann, C.J., and Cohen, S.M. (1996). Distinct mitogenic and cell fate specification functions of *wingless* in different regions of the wing. Development *122*, 1781–1789.

Noordermeer, J., Johnston, P., Rijsewijk, F., Nusse, R., and Lawrence, P.A. (1992). The consequences of ubiquitous expression of the wingless gene in the Drosophila embryo. Development *116*, 711–719.

Nusse, R., and Varmus, H.E. (1992). Wnt genes. Cell *69*, 1073–1087.

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. Nature *287*, 795–801.

Pan, D., and Rubin, G.M. (1995). cAMP-dependent protein kinase and *hedgehog* act antagonistically in regulating *decapentaplegic* transcription in Drosophila imaginal discs. Cell *80*, 543–552.

Parkin, N.T., Kitajewski, J., and Varmus, H.E. (1993). Activity of Wnt-1 as a transmembrane protein. Genes Dev. *7*, 2181–2193.

Peifer, M., Rauskolb, C., Williams, M., Riggleman, B., and Wieschaus, E. (1991). The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. Development *111*, 1029–1043.

Peifer, M., and Wieschaus, E. (1990). The segment polarity gene

armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. Cell *63*, 1167–1176.

Phillips, R.G., and Whittle, J.R.S. (1993). *wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. Development *118*, 427–436.

Pignoni, F., Balderelli, R.M., Steingrimsson, E., Diaz, R.J., Patapoutian, A., Merriam, J.R., and Lengyel, J.A. (1990). The Drosophila gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. Cell *62*, 151–163.

Roelink, H. (1995). Tripartite signaling of pattern: interactions between Hedgehogs, BMPs and Wnts in the control of vertebrate development. Curr. Opin. Neurobiol. *6*, 33–40.

Roth, S., Stein, D., and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorso-ventral pattern in the Drosophila embryo. Cell *59*, 1189–1202.

Rulifson, E.J., and Blair, S.S. (1995). *Notch* regulates *wingless* expression and is not required for reception of the paracrine *wingless* signal during wing margin neurogensis in *Drosophila*. Development *121*, 2813–1824.

Rushlow, C.A., Han, K., Manley, J.L., and Levine, M. (1989). The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in Drosophila. Cell *59*, 1165–1177.

Slack, J.M.W. (1987). Morphogenetic gradients—past and present. Trends Biochem. *12*, 200–204.

Spemann, H. (1938). Embryonic Development and Induction (New Haven, Connecticut: Yale University Press).

Spradling, A.C., Stern, D.M., Kiss, I., Roote, J., Laverty, T., and Rubin, G.M. (1995). Gene disruptions using P transposable elements: an integral component of the Drosophila genome project. Proc. Natl. Acad. Sci. USA *92*, 10824–10830.

Steward, R. (1989). Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell *59*, 1179–1188.

Struhl, G., and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. Cell *72*, 527–540.

Treisman, J.E., and Rubin, G.M. (1995). *wingless* inhibits morphogenetic furrow movement in the *Drosophila* eye disc. Development *121*, 3519–3527.

Turing, A. (1952). The chemical basis of morphogenesis. Philos. Trans. R. Soc. Lond. (B) *237*, 37–72.

van-den-Heuvel, M., Nusse, R., Johnston, P., and Lawrence, P.A. (1989). Distribution of the wingless gene product in Drosophila embryos: a protein involved in cell–cell communication. Cell *59*, 739–49.

Vincent, J. (1994). Morphogens dropping like flies? Trends Genet. *10*, 383–385.

Vincent, J., and Lawrence, P.A. (1994). Drosophila *wingless* sustains *engrailed* expression only in adjoining cells: evidence from mosaic embryos. Cell *77*, 909–915.

Wieschaus, E., and Riggleman, R. (1987). Autonomous requirements for the segment polarity gene armadillo during Drosophila embryogenesis. Cell *49*, 177–84.

Wilder, E.L., and Perrimon, N. (1995). Dual functions of *wingless* in the *Drosophila* leg imaginal disc. *121*, 477–488.

Williams, J.A., Bell, J.B., and Carroll, S.B. (1991). Control of *Drosophila* wing and haltere development by the nuclear *vestigial* gene product. Genes Dev. *5*, 2481–2495.

Williams, J.A., Paddock, S.W., and Carroll, S.B. (1993). Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete subregions. Development *117*, 571–584.

Wilson, E.G. (1925). The Cell in Development and Inheritance (New York, New York: The Macmillan Company).

Wilson, I.A., Niman, H.L., Houghten, R.A., Cherenson, A.R., Connolly, M.L., and Lerner, R.A. (1984). The structure of an antigenic determinant in a protein. Cell *37*, 767–778.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development *117*, 1223–1237.

Yost, C., Torres, M., Miller, J.R., Huang, E., Kimmelman, D., and Moon, R.T. (1996). The axis-inducing activity, stability and subcellular distribution of β-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev. *10*, 1443–1454.

Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of *engrailed*, *hedgehog*, and *decapentaplegic* in the *Drosophila* wing. Development *121*, 2265–2278.

Note Added in Proof

Lawrence et al. (1996) report independent evidence that different levels of Wg can specify distinct cellular outputs: Lawrence, P.A., Sanson, B., and Vincent, J.-P. (1996). Compartments, wingless and engrailed–patterning the ventral epidermis of Drosophila embryos. Development, in press.