Developmental Biology **235,** 467–475 (2001)
doi:10.1006/dbio.2001.0320, available online at http://www.idealibrary.com on IDEAL®

Wingless Capture by Frizzled and Frizzled2 in Drosophila Embryos

Magalie Lecourtois, Cyrille Alexandre, Laurence Dubois, and Jean-Paul Vincent[1]

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

A variety of factors could influence how far developmental signals spread. For example, the Patched receptor limits the range of its ligand Hedgehog. Somehow, the Frizzled2 receptor has the opposite effect on its ligand. Increasing the level of Frizzled2 stabilizes Wingless and thus extends the Wingless gradient in *Drosophila* wing imaginal disks. Here we ask whether Frizzled or Frizzled2 affects the spread of Wingless in *Drosophila* embryos. We show that in the embryonic epidermis, the combined expression of both receptors is lowest in the *engrailed* domain. This is because expression of Frizzled is repressed by the Engrailed transcription factor, whereas that of Frizzled2 is repressed by Wingless signaling. Receptor downregulation correlates with an early asymmetry in Wingless distribution, characterized by the loss of Wingless staining in the *engrailed* domain. Raising the expression of either Frizzled or Frizzled2 in this domain prevents the early disappearance of Wingless-containing vesicles. Apparently, Wingless is captured, stabilized, and quickly internalized by either receptor. As far as we can tell, captured Wingless is not passed on to further cells and does not contribute to the spread of Wingless. Receptor downregulation in the posterior compartment may contribute to dampening the signal at the time when cuticular fates are specified.

Key Words: **Drosophila embryos; Wingless transport; Wingless stability; Engrailed; Frizzled; Frizzled2; epidermal pattern; signal modulation.**

INTRODUCTION

During pattern formation, cells convey signals to one another with a relatively small number of secreted proteins. For any given signal, the range of action seems to depend on the local context. For example, in third instar *Drosophila* wing imaginal disks, the Wingless protein spreads and acts symmetrically from its source over a range of approximately 25 cells (Cadigan *et al.*, 1998; Strigini and Cohen, 2000), whereas in embryos, the range is 5 cells at most (Sanson *et al.*, 1999). In principle the range of a signal could be modulated by the rate of secretion, the composition of the extracellular matrix, the amount of receptor, the rate of internalization, or the time allowed for diffusion. Among these factors, only the level of receptor has so far been clearly documented to affect the range of its corresponding ligand. Patched is a receptor for the secreted signal encoded by *hedgehog* and if the Hedgehog-binding domain of Patched is mutated, the spread of Hedgehog within wing imaginal disks increases (Chen and Struhl, 1996). This effect is consistent with the idea that excess receptor soaks up the ligand and prevents it from spreading (see Kerszberg and Wolpert, 1998 for a formal analysis). Surprisingly, the opposite is apparently seen for Wingless and its receptor Frizzled2. When Frizzled2 is overexpressed in wing imaginal disks, the level of detectable Wingless protein increases over a broad domain. This expansion is reflected in target gene expression. For example, expression of *achaete*, a "high signaling" target widens from one to three cell diameters on either side of the D/V boundary (Cadigan *et al.*, 1998).

Increased Frizzled2 could extend the Wingless gradient in two ways. One is that, as its expression increases, Frizzled2 captures more Wingless from the extracellular space and protects it from degradation (Cadigan *et al.*, 1998). As a result, the number of ligand–receptor complexes would increase, leading to increased signaling at all locations

[1] To whom correspondence should be addressed at Medical Research Council, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK. Fax: 44 (0) 20 8913-8543. E-mail: jp.vincent@nimr.mrc.ac.uk.

0012-1606/01 $35.00

where Wingless is normally available in the extracellular space. In addition, locally stabilized Wingless could spill over, thus extending the range of Wingless beyond its normal reach. Although this second mechanism may seem implausible at first, one could imagine that, if Wingless were transported by planar transcytosis (e.g., Moline *et al.*, 1999), increased levels of Frizzled2 could increase the rate of receptor-mediated endocytosis and hence favor resecretion of Wingless toward more distant cells (like passing the baton in a relay race). Alternatively, the range could be extended along an extracellular route if the off rate of the receptor-ligand interaction is sufficiently high.

As shown previously (Martinez Arias, 1993; Sanson *et al.*, 1998), the range of Wingless in *Drosophila* embryos becomes asymmetric some time before Stage 11. We confirm that early in development, Wingless is readily detected throughout the domain of *engrailed* expression (that lies at the posterior of the Wingless source). Then, during Stage 10, Wingless immunostaining is specifically lost in these cells (Fig. 1) because of either changes in transport efficiency or destabilization. Here we ask to what extent changes in the expression of the Frizzled receptors might contribute to this early asymmetry in Wingless distribution.

In agreement with earlier immunofluorescence data (Bhanot *et al.*, 1996; Müller *et al.*, 1999), we show that, in the embryonic epidermis, the combined transcription of the two *frizzled* genes is lowest in the *engrailed* domain. Expression of each *frizzled* is regulated by distinct mechanisms: Engrailed represses *frizzled* expression, whereas *frizzled2* expression is repressed by Wingless signaling. Downregulation of both Frizzled receptors in the *engrailed* domain is likely to play a role in reducing Wingless levels there because we find that ectopic expression of *frizzled* or *frizzled2* restores a high level of detectable Wingless protein. We find no evidence of the sustained signal being passed on to further cells in a non-cell-autonomous manner: local increase of frizzled2 expression does not extend how far Wingless can spread. We conclude that downregulation of both Frizzled receptors has no effect on Wingless transport per se but it does decrease Wingless stability and thus contributes to decreasing the sensitivity of *engrailed*-expressing cells to Wingless available in the extracellular space.

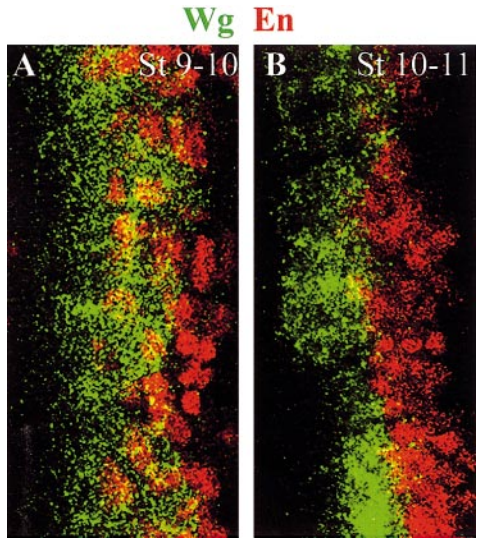


FIG. 1. Wingless staining is lost from the *engrailed* domain at Stage 10. Wild type embryos of Stage 9 (A) and late Stage 10 (B) stained with anti-Wingless and anti-Engrailed. Although many Wingless-containing vesicles are detected in the *engrailed* domain at Stage 9, very few can be seen at Stage10.

MATERIALS AND METHODS

Drosophila Stocks

wgCX4 (Baker, 1987) and *enCX1* (Heemskerk *et al.*, 1991) are presumed null alleles (see Flybase at http://flybase.bio.indiana.edu/). The following transgenic stocks were used: *engrailed-Gal4* (A. Brand, Welcome Institute, Cambridge, UK), *armadillo-Gal4* and *armadillo-Gal4VP16* (Sanson *et al.*, 1996), *UAS-frizzled* and *UAS-frizzled2* (Zhang and Carthew, 1998), *paired-Gal4* (Bloomington Stock Center), *UAS-engrailed* (Guillen *et al.*, 1995), *UAS-VP16-Engrailed* (Alexandre *et al.*, manuscript in preparation) and *UAS-wingless* (Lawrence *et al.*, 1995). The *ftz-Gal4* strain was made for the purpose of this study. Briefly, the whole 5′ regulatory region of *ftz* including the promoter (6.6 kb as defined by Hiromi and Gehring, 1987) was isolated. The two internal *Bam*HI sites were removed by digestion followed by filling in and religation. The modified control region was then inserted (as a *Kpn*I–*Bam*HI fragment) upstream of *Gal4* in pGaTB (flybase.bio.indiana.edu). From this new plasmid, a *Kpn*I–*Not*I fragment (comprising the *ftz* regulatory region and the *gal4* coding region) was excised and inserted in P{CaSpeR-4} (flybase.bio.indiana.edu).

Embryo Preparations

Immunofluorescence was done according to standard protocols (e.g., Vincent and O'Farrell, 1992) using Alexa fluorescent conjugates (Alexa 488 and Alexa 592; Molecular Probes. Eugene, OR). Antibodies used were rabbit anti-Engrailed (gift from C. H. Girdham and P. O'Farrell, UCSF), mouse anti-Engrailed (4D9), and mouse anti-Wingless (4D4) both from the Developmental Studies Hybridoma Bank.

RNA *in situ* hybridization and double antibody/RNA labeling were done as described by Alexandre *et al.* (1999). The following cDNA were used: *engrailed* (Poole *et al.*, 1985), *wingless* (gift from N. Parkin, Aviron Inc., Mountain View, CA), *frizzled*, and *frizzled2* (both from R. Nusse, Stanford University).

RESULTS

Regulation of frizzled and frizzled2 Expression

Both Frizzled and Frizzled2 proteins are expressed in a dynamic fashion during the first 12 h of development. In particular, the level of Frizzled is down in the *engrailed* domain (Müller *et al.*, 1999) and Frizzled2 is relatively less abundant in the apparent domain of Wingless action (Bh-

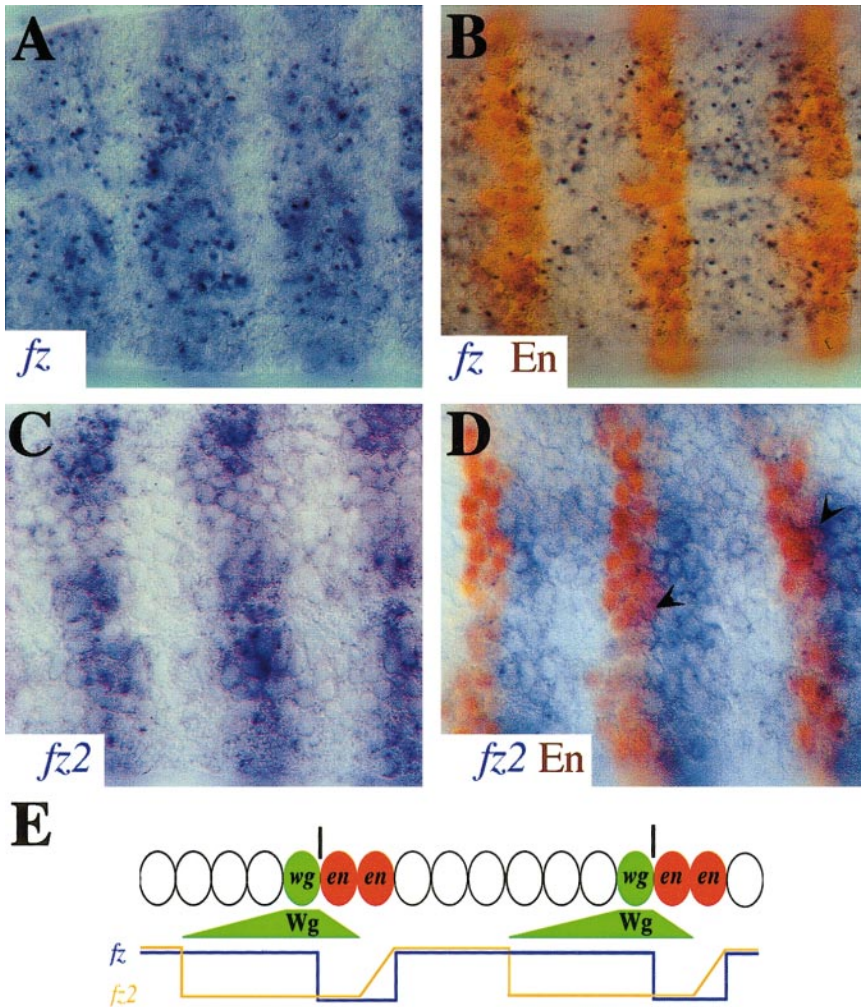


FIG. 2. Combined expression of *frizzled* and *frizzled2* is lowest in the *engrailed*-expressing cells. (A–D) Posterior portion of wild type Stage 10 embryos (germ band extended). (A) *In situ* hybridization analysis with a *frizzled* probe shows that *frizzled* is expressed in segmental stripes. (B) Double staining with anti-Engrailed in ochre and a *frizzled* RNA probe in purple reveals that *frizzled* is expressed in all cells except those expressing *engrailed*. (C) *In situ* hybridization analysis using a *frizzled2* probe shows a periodic pattern of expression. (D) The *frizzled2* domain (RNA probe in purple) is posterior to the *engrailed* domain (anti-Engrailed in ochre) with occasional overlap in the most posterior *engrailed*-expressing cells (indicated by arrowheads). (E) Schematic diagram representing the pattern of expression of *wingless*, *engrailed*, *frizzled*, and *frizzled2* and the spatial distribution of detectable Wingless protein in the ventral epidermis of early Stage 11 embryos. *engrailed* (red circles) is expressed in the two rows of cells at the posterior boundary of each segment (black lines above cells represent parasegment borders), and *wingless* (green circles) is expressed in a single row of cells just anterior to the *engrailed* expression domain. The green triangle shows the asymmetric distribution of Wingless protein from its source at Stage 11. Note that combined expression of *frizzled* (blue line) and *frizzled2* (ochre line) is lowest (H: high; L: low) in *engrailed*-expressing cells, especially the most anterior ones.

anot *et al.*, 1996). We have studied in detail the patterns of transcription around Stages 8 and 11 (3.5–7 h AEL). Although *frizzled* expression is initially uniform during gastrulation, it begins to resolve into a periodic pattern by Stage 9 (4 h AEL; Fig. 2A). Double staining shows that, at Stage 10 (4.5–5 h AEL), *frizzled* transcripts are abundant in all cells except those that express *engrailed* (Fig. 2B). Expression of *frizzled2* also becomes segmental around Stage 9, a pattern that is clearly marked at Stage 10 (Fig. 2C): broad stripes of *frizzled2* expression are detected at the posterior of each *engrailed* stripe (Fig. 2D). Thus, at Stage 10 (4.5–5 h AEL), combined expression of *frizzled* and *frizzled2* is lowest in *engrailed*-expressing cells, especially those nearest to the source of Wingless (Fig. 2E). Note, however, that residual mRNA remains, possibly as a result of maternal contribution or low-level zygotic transcription.

As shown above, *frizzled* is downregulated in cells that express Engrailed, a known transcriptional repressor (Jaynes and O'Farrell, 1988; Han *et al.*, 1989). We now ask whether Engrailed could be a direct repressor of *frizzled* expression. Consistent with this possibility, in *engrailed* mutant embryos, *frizzled* is expressed ubiquitously (compare Figs. 3A and 3B). Conversely, overexpression of *engrailed* with the uniform *armadillo-Gal4* driver leads to complete repression in abdominal segments (Figs. 3C and 3D), while ectopic expression with the *paired-Gal4* driver abrogates *frizzled* expression in a pair-rule fashion (Fig. 3E). Thus both gain- and loss-of-function experiments suggest that Engrailed represses *frizzled* expression. To confirm this suggestion, we used a form of Engrailed that was converted into a transcriptional activator by replacing its repressor domain with the activation domain of the Herpes virus protein VP16 (*UAS-VP16-En*). This fusion protein activates *cubitus interruptus* (Alexandre and Vincent, manuscript in preparation), a gene known to be repressed by Engrailed. We find that expression of this fusion protein in the *engrailed* domain (*engrailed-gal4, UAS-VP16-En*) leads to ectopic activation of *frizzled* transcription and thus abrogates segmental modulation (Fig. 3F). It is therefore likely that, in embryos, Engrailed is a direct repressor of *frizzled* expression.

In wing imaginal disks, Wingless signaling represses *frizzled2* transcription (Cadigan *et al.*, 1998). As seen in Fig. 2, embryonic expression of *frizzled2* is low in cells that are known to be under the influence of Wingless (diagram in Fig. 2), suggesting that the regulatory interaction seen in disks holds in the embryo. Indeed, as shown in Fig. 4B, *frizzled2* is ubiquitously expressed in *wingless* mutant embryos (segmental modulation remains, presumably because of the existence of additional, minor regulators). Therefore, Wingless is needed for segmental repression of *frizzled2*. Conversely, ectopic expression of *wingless* with the uniform *armadillo-Gal4* driver abolishes *frizzled2* expression (Fig. 4C). Thus as in imaginal disks, Wingless signaling represses *frizzled2* transcription in the embryonic epidermis.

Increased Expression of frizzled or frizzled2 Alters the Distribution of Wingless

If the loss of Wingless protein that occurs around Stage 10 in the *engrailed* domain is the result of the transcriptional downregulation of *frizzled* and *frizzled2* (which happens around the same time), then adding back the receptors would be expected to restore Wingless immunostaining. Indeed, in Stage 11 embryos that overexpress *frizzled* with the *engrailed-Gal4* driver, many Wingless-containing vesicles are detected throughout the domain of *engrailed* expression, especially at ventral positions (Fig. 5B). For comparison, Wingless-containing vesicles are rarely detected in the *engrailed* domain of similarly staged wild type embryos. The prolonged presence of Wingless is not the result of ectopic *wingless* transcription (compare Figs. 5D and 5E). Therefore sustained Wingless protein originates from the normal domain of expression. (Note that Frizzled does not increase Wingless staining in imaginal disks (Cadigan *et al.*, 1998; Rulifson *et al.*, 2000)). In embryos carrying *engrailed-Gal4, UAS-frizzled2,* Wingless protein is also maintained within the domain of *engrailed* expression (Fig. 5C). Again, increased Wingless staining occurs at the protein level because no ectopic *wingless* transcription is seen (Fig. 5F).

In additional experiments, Frizzled was misexpressed with the *ftz-Gal4* driver, which is active in the *engrailed* domain of alternate segments, thus allowing internally controlled assessment of staining intensity. In these embryos (shown in Fig. 5G), detectable Wingless is abundant in the *ftz*-expressing *engrailed* domains. Interestingly, the amount of Wingless protein appears to decrease in the adjoining expressing cells (at the anterior) suggesting that overexpressed Frizzled may lead to a redistribution of limited amounts of Wingless (see Discussion).

Although receptor overexpression leads to increased Wingless staining, this situation is not sustained. At late Stage 11 (7 h AEL), the number of Wingless-containing vesicles in the *engrailed* domain of *engrailed-Gal4 UAS-frizzled2* drops back down to wild type levels (not shown), indicating the existence of an additional mechanism that destabilizes Wingless (Dubois *et al.*, 2001). Nevertheless, during Stages 9 and 10 (4–5.5 h AEL), either Frizzled or Frizzled2 is sufficient to maintain a high amount of detectable Wingless in the domain of *engrailed* expression.

Captured Wingless Is Not Passed on to More Distant Cells

In imaginal disks, a truncated form of Frizzled2 [the extracellular domain linked to a glycosyl–phosphatidyl inositol (GPI) anchor; Δ*frizzled2-GPI*] not only stabilizes Wingless but also passes it on to neighboring cells (Cadigan *et al.*, 1998). Does capture of Wingless by the full-length receptors similarly lead to an extension of its spread (i.e., bring Wingless where there was not any previously)? Figures 5B and 5C suggest it does not but immunofluorescence could fail to detect faint signals and therefore, we used a functional assay (the expression of target genes) to detect active Wingless. In the wild type, at mid-Stage 11 (6.5 h AEL), *engrailed* stripes are two to four cells wide (Fig. 6A). We know that more posterior cells could respond if they received Wingless, given that ubiquitous Wingless broadens *engrailed* stripes (Noordermeer *et al.*, 1992). Likewise, in *engrailed-Gal4, UAS-Wingless* embryos, *engrailed* stripes broaden progressively because the spread of Wingless is artificially increased. At the end of broadening (when the *engrailed* promoter becomes impervious to Wingless signaling, around Stage 11), *engrailed* stripes have become 5 to 6 cells wide (Fig. 6B) and expression of *rhomboid* begins, pushed back by as many cells. Thus, *engrailed-Gal4, UAS-Wingless* embryos serve as a positive control for the effect of extending the spread of Wingless. In embryos expressing

frizzled or *frizzled2* ectopically, we found no significant expansion of the *engrailed* expression domain (Fig. 6C). Of course subtle expansion would be hard to detect but, for comparison, *achaete* expression widens by a factor of three in imaginal disks overexpressing Frizzled2 (Cadigan and Nusse, 1998). Thus, in our embryonic assay, captured Wingless is not passed on to further cells.

Excessive Expression of frizzled or frizzled2 Increases Wingless Signaling in a Wingless-Dependent Manner

Even though the level of Frizzled receptors is lowest in the *engrailed* domain, some activity must remain (maybe from maternal stores or low-level expression) because sustained *engrailed* expression requires Wingless signaling (DiNardo *et al.*, 1988; Martinez-Arias *et al.*, 1988). But why is the expression of the Frizzled receptors downregulated in the *engrailed* domain? Increasing expression of *frizzled* or *frizzled2* with the *engrailed-Gal4* driver leads to partial loss of the first row of denticles in each segment (Figs. 7A–7C), an indication of increased Wingless signaling. A priori, this may or may not depend on the Wingless ligand. Experiments with imaginal disks have suggested that *frizzled2* overexpression does not overcome the need for Wingless (Cadigan *et al.*, 1998). To ask whether this is also true for *frizzled*, we created *wingless* mutant embryos overexpressing *frizzled* in the *engrailed* domain (*wg*[*CX4*] *engrailed-Gal4 UAS-frizzled*). The cuticle phenotype of these embryos is indistinguishable from that of *wingless* mutants (data not shown). Because, in the presence of the normal Wingless source, *frizzled* overexpression can cause cells fated to secrete row 1 denticle to make bald cuticle, it appears that Wingless does normally reach these cells and that an increase in receptor expression allows them to respond. Note that the loss of denticles induced by Frizzled overexpression is not fully penetrant. Some *engrailed*-expressing cells still make denticles despite massive overexpression (*engrailed-Gal4* is a strong driver). A subsequent degradation mechanism may explain why overexpression of the receptor has only an incomplete effect on cuticular fate. Intriguingly, loss of row 1 denticles is more penetrant with Frizzled (over 80% of denticle belts have missing row 1 denticles), even though its affinity for Wingless is lower than that of Frizzled2 (Rulifson *et al.*, 2000). Perhaps degradation of Frizzled2/Wingless complexes is more rapid than that of Frizzled/Wingless.

DISCUSSION

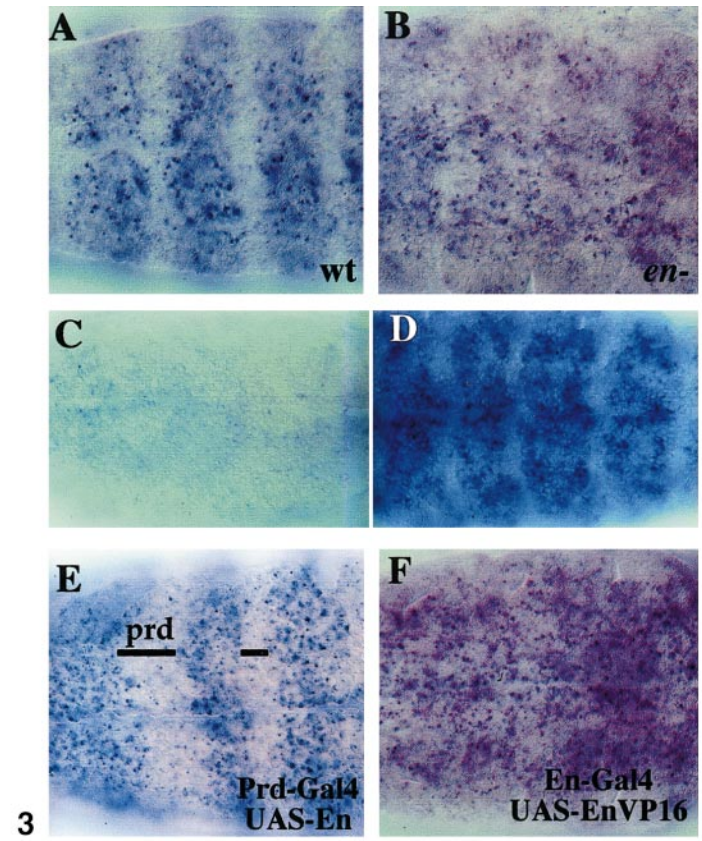
Transcription Downregulation of frizzled and frizzled2 in the engrailed Domain

At Stage 10 of *Drosophila* embryogenesis, the amount of detectable Wingless decreases within the *engrailed* domain. This corresponds to the time when both *frizzled* and *frizzled2* are transcriptionally downregulated there. As we have shown, artificially increasing the expression of *frizzled* or *frizzled2* prevents the early loss of Wingless staining; binding of Wingless to its receptors may render it inaccessible to extracellular proteases. This suggests that, in the wild type, transcriptional downregulation of the receptors causes the early loss of Wingless immunostaining. Two distinct mechanisms repress the transcription of *frizzled* and *frizzled2*: Engrailed itself appears to repress *frizzled*, whereas Wingless signaling represses *frizzled2*. Repression of *frizzled* expression by Engrailed is not seen in imaginal disks where, presumably, a cofactor is missing. In contrast, repression of *frizzled2* by Wingless signaling appears to be a general feature (Cadigan *et al.*, 1998). As a result of two distinct repression mechanisms, the combined expression of *frizzled* and *frizzled2* is lowest in the *engrailed* cells, especially those nearest to the source of Wingless. Nevertheless, residual activity must remain because *engrailed*-expressing cells respond to Wingless as late as 8.5 h AEL (Dougan and DiNardo, 1992), whereas the complete absence of *frizzled* and *frizzled2* activity phenocopies a *wingless* null mutation (Bhanot *et al.*, 1999; Chen and Struhl, 1999).

Spread vs. Range

An increased level of Frizzled converts prospective row 1-secreting cells to the bald fate (Fig. 7). Given that overexpression of *frizzled* does not activate the pathway in the absence of Wingless, it follows that, at the time of denticle specification, Wingless reaches prospective row 1 cells but it is not normally effective there because receptor levels are too low and also because of the antagonizing influence of EGFR signaling. Thus, in this instance, there is a difference between spread of the protein and range of action.

Cadigan *et al.* (1998) suggested that overexpressed Frizzled2 extends the Wingless gradient by stabilizing existing Wingless. Could it also be that stabilized Wingless be passed on to further cells? In apparent support for this possibility, expression of Δ*frizzled2-GPI* does cause a nonautonomous increase in Wingless staining both in imaginal disks (Cadigan et al.,1998) and in embryos (Dubois *et al.*, 2001). However, no similar effect has been documented with the full-length receptors. In both imaginal disks and embryos, receptor overexpression raises the detectability of Wingless only in cells that are normally within reach (see Cadigan *et al.*, 1998 for disks and Fig. 5 for embryos). In the embryo, this corresponds to the *engrailed* domain and, as shown in Fig. 7, no broadening of *engrailed* expression occurs in response to receptor overexpression, suggesting that overexpressed Frizzled or Frizzled2 does not extend the range of Wingless. Whether receptor overexpression restricts the range of Wingless as would be expected if they sequestered the ligand is not clear, although Fig. 5G suggests that increased capture in receiving cells may reduce Wingless levels in secreting cells (in a nonautonomous manner). Because GPI-anchored proteins could possibly transfer from cell to cell (Kooyman *et al.*, 1995), it may be



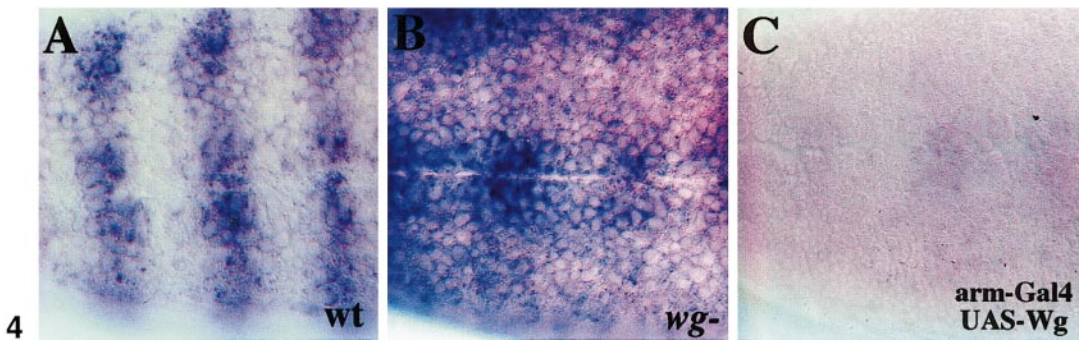
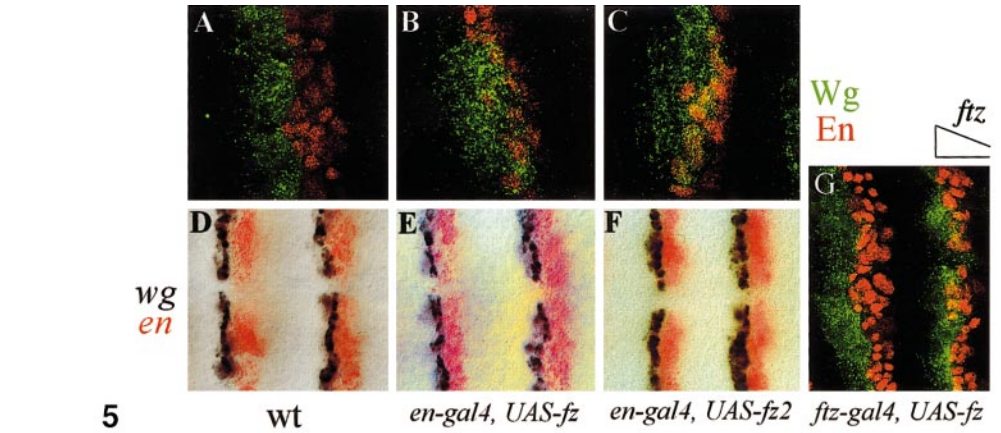
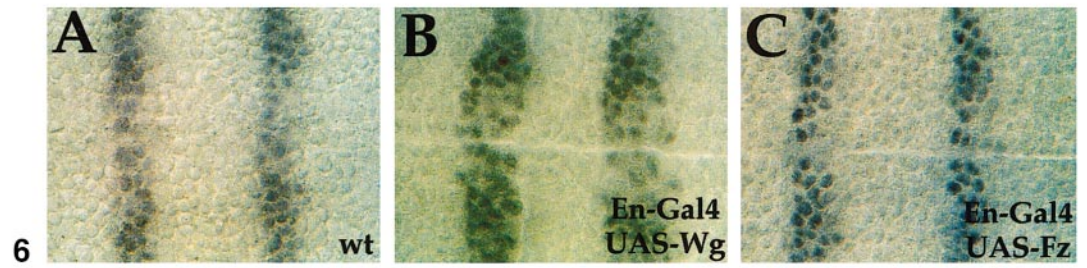


FIG. 3. Engrailed represses *frizzled* expression. (A) *In situ* hybridization of Stage 10 embryos with a *frizzled* RNA probe. (B) In an *engrailed* null mutant, *frizzled* is no longer repressed segmentally. (C and D) Ubiquitous expression of Engrailed with the *armadillo-gal4* driver leads to repression of *frizzled* expression throughout the abdomen. Shown are sibling embryos treated and photographed identically (C shows arm-Gal4 UAS-engrailed, whereas D shows a control arm-gal4 embryos). (E) Ectopic expression of Engrailed in the *paired* domain (a broad region including the *engrailed* domain and more anterior cells in alternate segment; long black line) leads to increased repression of *frizzled* expression in a pair-rule fashion. (F) Overexpression of an "activated" form of Engrailed (VP16-En) in the *engrailed* domain relieves *frizzled* repression there. We know from unpublished work that VP16-En *activates* ectopic *wingless* expression (Alexandre *et al.*, manuscript in preparation). However, in *engrailed* mutants, which lose *wingless* expression, *frizzled* is also ectopically expressed (see above). Therefore, repression of *frizzled* by Engrailed is not indirectly mediated by an effect on *wingless* expression and it appears that Wingless does not regulate *frizzled* expression.

FIG. 4. Wingless signaling represses *frizzled2* transcription. (A) *In situ* hybridization of a Stage 10 embryo with a *frizzled2* RNA probe. (B) In a *wingless* null mutant, *frizzled2* is derepressed, although still somewhat modulated segmentally. (C) Ubiquitous overexpression of *wingless* in embryos, using the uniform *armadillo-Gal4* driver, eliminates *frizzled2* expression.





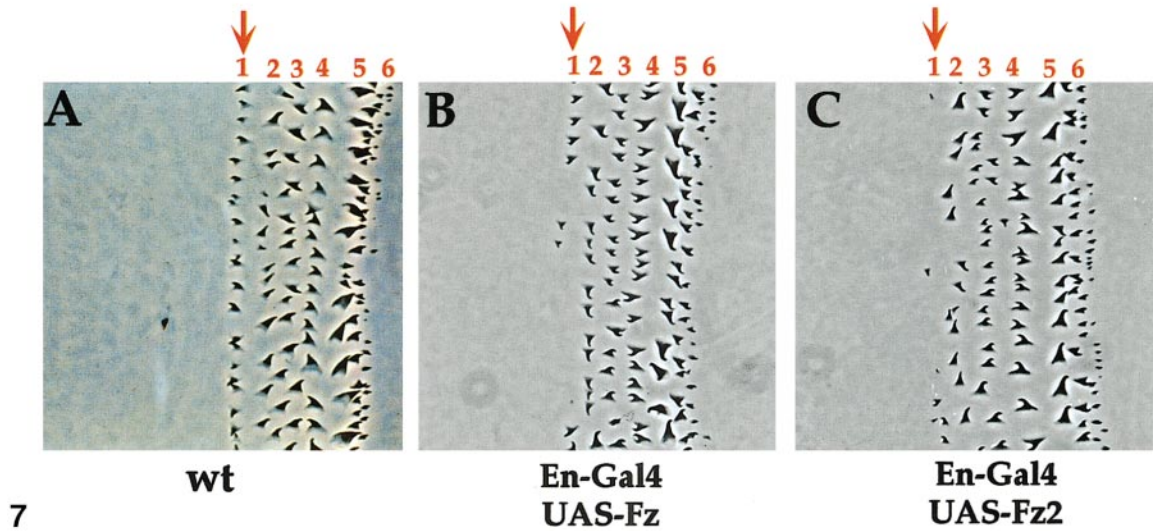


FIG. 5. Ectopic expression of *frizzled* or *frizzled2* leads to increased Wingless staining in receiving cells. (A–C) Single hemisegments from the posterior portion of early Stage 11 embryos double-stained with anti-Wingless (in green) and anti-Engrailed (in red). (D–F) Pairs of segments from the same embryonic region stained with a *wingless* RNA probe (in purple) and an *engrailed* RNA probe (in red). Overexpression of *frizzled* (B) or *frizzled2* (C) in the *engrailed* domain leads to increased accumulation of Wingless-containing vesicles compared to that of wild type (A). This is not the result of ectopic *wingless* expression in *engrailed*-expressing cells: expression of *wingless* in *engrailed-Gal4, UAS-frizzled* (E), or *engrailed-Gal4, UAS-frizzled2* (F) embryos are similar to that observed in wild type embryos (D). Note that Frizzled2 overexpression with *engrailed-gal4* leads to serious, generalized defects in about half of the embryos (not shown). The embryo shown in F is from the ones that were relatively unaffected. Misexpression of frizzled (G) with the *ftz-gal4* driver leads to increased Wingless staining in alternate posterior compartments while apparently restricting the width of adjacent Wingless expression stripes. In this and subsequent immunofluorescence pictures are stacks of confocal images.

FIG. 6. Ectopic expression of *frizzled* or *frizzled2* does not affect how far Wingless spreads. Posterior portion of early Stage 11 embryos stained with an anti-Engrailed antibody. (A) In wild type embryos, the width of Engrailed stripes is about 2 to 3 cells wide. (B) In *engrailed-Gal4, UAS-Wingless* embryos, Engrailed stripes broaden to 5–6 cells because Wingless is made to spread further posteriorly (see Sanson *et al.*, 1999). (C) In contrast *frizzled* overexpression in the *engrailed* domain (*engrailed-Gal4 UAS-frizzled*) does not expand the Engrailed stripe, indicating that Frizzled does not affect the spread of Wingless.

that the increased spread seen in response to Δ*Frizzled2–GPI* expression in disks is an artifact of labile membrane association by a GPI anchor.

We previously suggested that the spread of Wingless might be specifically retarded in the *engrailed* domain, in that immunostaining decreases in these cells around Stage 10 (Sanson *et al.*, 1999). In fact, our original expectation at the outset of this work was that the relatively low combined expression of *frizzled* and *frizzled2* in the *engrailed* domain would be instrumental in slowing down the spread of Wingless. However, as indicated above, increasing the expression of *frizzled* or *frizzled2* does not extend the range of Wingless action during Stages 9 and 10. Therefore, we suggest the Frizzled receptors do no contribute positively to Wingless transport per se. However, because they stabilize or capture Wingless, the receptors do influence the distribution of the signal. Antibody staining suggests that, initially, the range of Wingless is not distinctly asymmetric (Martinez Arias, 1993). This is reflected in the expression of target genes around Stage 11. At this stage, *engrailed* is activated *posteriorly* over 2–4 cell diameters and *rhomboid* expression is repressed in the same domain. At the same stage, the posterior edge of each *serrate* stripe, which marks the *anterior* edge of the range of Wingless (Alexandre *et al.*, 1999), is located roughly 3–4 cell diameters from the source of Wingless (C. Alexandre, unpublished observations). The fact that anteriorward cell spreading contributes to the forward spread of Wingless (Pfeiffer *et al.*, 2000) may account for the slight difference between anterior and posterior spread. Therefore, until early Stage 10, the spread of Wingless is symmetric (with consequences on target gene expression extending into Stage 11).

Signal Downregulation

Our results suggest that downregulation of the Frizzled receptors reduces the spread of Wingless into the posterior compartment, not by affecting its transport but rather by reducing its stability. This would lead to a reduced number of effective receptor–ligand complexes and hence dampened signaling. We expect this to commence during Stage 10. Transcriptional repression of receptor expression has been shown to contribute to dampening of signaling in other instances such as the α1-adrenergic receptor (Izzo *et al.*, 1990). In this and many other examples, additional strategies such as desensitization are also at work (reviewed in Böhm *et al.*, 1997). Likewise, additional mechanisms for dampening Wingless signaling are likely to exist. Indeed, we found that, after Stage 11, residual Wingless/receptor complexes are rapidly degraded (and hence rendered ineffective) in prospective denticle-secreting cells (Dubois *et al.*, 2001). This targeted degradation of Wingless can account for the fact that row 1 denticles still form in embryos that massively express *frizzled* or *frizzled2* (Fig. 7). Both mechanisms of signal downregulation (repression of receptor transcription and degradation of receptor/ligand complexes) dampen the action of Wingless toward the posterior, although more work is needed to assess their relative importance. Another outstanding issue is whether Frizzled and Frizzled2 are equivalent with respect to signal downregulation. Clearly, these receptors differ in terms of affinity for the ligand (Rulifson *et al.*, 2000). It may also be that differences in intracellular trafficking lead to distinct effects on Wingless signal downregulation.

ACKNOWLEDGMENTS

We thank the Bloomington Stock Center, R. Nusse, and R. Carthew for fly strains and the Developmental Studies Hybridoma Bank at the University of Iowa for antibodies. Thanks also to Roel Nusse for comments on the manuscript. M.L. is the recipient of a postdoctoral fellowship from the European Molecular Biology Organization; J.-P.V., C.A., and L.D. are supported by the UK Medical Research Council.

REFERENCES

Alexandre, C., Lecourtois, M., and Vincent, J.-P. (1999). Wingless and Hedgehog pattern Drosophila denticle belts by regulating the production of short-range signals. *Development* **126,** 5689–5698.

Baker, N. E. (1987). Molecular cloning of sequences from *wingless*, a segment polarity gene in Drosophila, and the spatial distribution of a transcript in embryos. *EMBO J.* **6,** 1765–1774.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the *frizzled* family from Drosophila functions as a Wingless receptor. *Nature* **382,** 225–230.

Bhanot, P., Fish, M., Jemison, J. A., Nusse, R., Nathans, J., and Cadigan, K. M. (1999). Frizzled and DFrizzled-2 function as redundant receptors for Wingless during Drosophila embryonic development. *Development* **126,** 4175–4186.

Böhm, S. K., Grady, E. F., and Bunnett, N. W. (1997). Regulatory mechanisms that modulate signaling by G-protein-coupled receptors. *Biochem. J.* **322,** 1–18.

Cadigan, K. M., Fish, M. P., Rulifson, E. J., and Nusse, R. (1998). Wingless repression of Drosophila *frizzled 2* expression shapes the Wingless morphogen gradient in the wing. *Cell* **93,** 767–777.

Chen, C., and Struhl, G. (1999). Wingless transduction by the Frizzled and Frizzled2 proteins of Drosophila. *Development* **126,** 5441–5452.

FIG. 7. Ectopic expression of *frizzled* or *frizzled2* increases the sensitivity of *engrailed*-expressing cells to Wingless. Wild type cuticular pattern in an abdominal segment (A). Note the stereotypical shape and polarity of denticles types, numbered 1–6). In *engrailed-Gal4, UAS-Frizzled* (B) and *engrailed-Gal4, UAS-Frizzled2* (C) embryos lose type 1 denticles (those secreted by the most posterior *engrailed* cells). This is seen in all embryos, although, at times, one or two segments remain unaffected. Thus, ectopic expression of *frizzled* or *frizzled2* in the posterior cells of each *engrailed* stripes converts them to the bald fate.

Chen, Y., and Struhl, G. (1996). Dual roles for Patched in sequestering and transducing Hedgehog. *Cell* **87,** 553–563.

DiNardo, S., Sher, E., Heemskerk-Jongens, 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.

Dougan, S., and DiNardo, S. (1992). Drosophila Wingless generates cell type diversity among *engrailed* expressing cells. *Nature* **360,** 347–350.

Dubois, L., Lecourtois, M., Alexandre, C., Hirst, J., and Vincent, J.-P. (2001). Regulated endocytic routing modulates Wingless signalling n Drosophila embryos. *Cell* **105,** 613–624.

Guillen, I., Mullor, J. L., Capdevila, J., Sanchez-Herrero, E., Morata, G., and Guerrero, I. (1995). The function of *engrailed* and the specification of Drosophila wing pattern. *Development* **121,** 3447–3456.

Han, K., Levine, M. S., and Manley, J. L. (1989). Synergistic activation and repression of transcription by Drosophila homeobox proteins. *Cell* **56,** 573–583.

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

Hiromi, Y., and Gehring, W. J. (1987). Regulation and function of the Drosophila segmentation gene *fushi tarazu*. *Cell* **50,** 963–974.

Izzo, N. J., Seidman, C. E., Colins, S., and Coluccci, W. S. (1990). a1-Adrenergic receptor mRNA level is regulated by norepinephrine in rabbit aortic smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **87,** 6268–6271.

Jaynes, J. B., and O'Farrell, P. H. (1988). Activation and repression of transcription by homeodomain-containing proteins that bind a common site. *Nature* **336,** 744–749.

Kerszberg, M., and Wolpert, L. (1998). Mechanisms for positional signaling by morphogen transport: A theoretical study. *J. Theor. Biol.* **191,** 103–114.

Kooyman, D. L., Byrne, G. W., McClellan, S., Nielsen, D., Tone, M., Waldmann, H., Coffman, T. M., McCurry, K. R., Platt, J. L., and Logan, J. S. (1995). In vivo transfer of GPI-linked complement restriction factors from erythrocytes to the endothelium. *Science* **269,** 89–92.

Lawrence, P. A., Bodmer, R., and Vincent, J.-P. (1995). Segmental patterning of heart precursors in Drosophila. *Development* **121,** 4303–4308.

Martinez Arias, A. (1993). Development and patterning of the larval epidermis of Drosophila. *In* "The Development of Drosophila melanogaster" (M. Bate and A. Martinez Arias, Eds.), pp. 517–608. Cold Spring Harbor Laboratory Press, Plainview, NY.

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.

Moline, M. M., Dierick, H. A., Southern, C., and Bejsovec, A. (2000). Non-equivalent roles of Drosophila Frizzled and Frizzled2 in embryonic Wingless signal transduction. *Curr. Biol.* **10,** 1127–1130.

Moline, M. M., Southern, C., and Bejsovec, A. (1999). Directionality of Wingless protein transport influences epidermal patterning in the Drosophila embryo. *Development* **126,** 4375–4384.

Müller, H., Samanta, R., and Wieschaus, E. (1999). Wingless signaling in the Drosophila embryo: Zygotic requirements and the role of the *frizzled* genes. *Development* **126,** 577–586.

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.

O'Keefe, L., Dougan, S. T., Gabay, L., Raz, E., Shilo, B. Z., and DiNardo, S. (1997). Spitz and Wingless, emanating from distinct borders, cooperate to establish cell fate across the Engrailed domain in the Drosophila epidermis. *Development* **124,** 4837–4845.

Pfeiffer, S., Alexandre, C., Calleja, M., and Vincent, J.-P. (2000). The progeny of *wingless*-expressing cells deliver the signal at a distance in Drosophila embryos. *Curr. Biol.* **10,** 321–324.

Piepenburg, O., Vorbrügge, G., and Jäckle, H. (2000). Drosophila segment borders result from unilateral repression of Hedgehog activity by Wingless signaling. *Mol. Cell.* **6,** 203–209.

Poole, S. J., Kauvar, L. M., Drees, B., and Kornberg, T. (1985). The *engrailed* locus of Drosophila: Structural analysis of an embryonic transcript. *Cell* **40,** 37–43.

Rulifson, E. J., Wu, C. H., and Nusse, R. (2000). Pathway specificity by the bifunctional receptor Frizzled is determined by affinity for Wingless. *Mol. Cell.* **6,** 117–126.

Sanson, B., Alexandre, C., Fascetti, N., and Vincent, J.-P. (1999). Engrailed and Hedgehog make the range of Wingless asymmetric in Drosophila embryos. *Cell* **98,** 207–216.

Sanson, B., White, P., and Vincent, J.-P. (1996). Uncoupling Cadherin-based adhesion from Wingless signaling in Drosophila. *Nature* **383,** 627–630.

Strigini, M., and Cohen, S. M. (2000). Wingless gradient formation in the Drosophila wing. *Curr. Biol.* **23,** 293–300.

Szuts, D., Freeman, M., and Bienz, M. (1997). Antagonism between EGFR and Wingless signaling in the larval cuticle of Drosophila. *Development* **124,** 3209–3219.

Vincent, J.-P., and O'Farrell, P. H. (1992). The state of *engrailed* expression is not clonally transmitted during early Drosophila development. *Cell* **68,** 923–931.

Zhang, J., and Carthew, R. W. (1998). Interactions between *wingless* and DFz2 during Drosophila wing development. *Development* **125,** 3075–3085.

Received for publication March 19, 2001
Revised April 27, 2001
Accepted May 7, 2001
Published online June 14, 2001