

Different mechanisms initiate and maintain *wingless* expression in the *Drosophila* wing hinge

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Accepted 6 June 2002

SUMMARY

The *Drosophila* gene *wingless* encodes a secreted signalling molecule that is required for many patterning events in both embryonic and postembryonic development. In the wing *wingless* is expressed in a complex and dynamic pattern that is controlled by several different mechanisms. These involve the Hedgehog and Notch pathways and the nuclear proteins Pannier and U-shaped. In this report, we analyse the mechanisms that drive *wingless* expression in the wing hinge. We present evidence that *wingless* is

initially activated by a secreted signal that requires the genes *vestigial*, *rotund* and *nubbin*. Later in development, *wingless* expression in the wing hinge is maintained by a different mechanism, which involves an autoregulatory loop and requires the genes *homothorax* and *rotund*. We discuss the role of *wingless* in patterning the wing hinge.

Key words: *Drosophila*, Pattern formation, *wingless*, Wing hinge

INTRODUCTION

Members of the Wnt family of secreted glycoproteins are involved in numerous developmental events in many organisms, from the nematode *C. elegans* to mammals. Among functions provided by Wnt proteins are embryonic patterning, cell fate specification, cell polarity, cell proliferation and pattern-organising activity (Cadigan and Nusse, 1997).

In *Drosophila* the best characterised Wnt gene is *wingless* (*wg*), one of the first members of this gene family to be identified (Sharma and Chopra, 1976; Baker, 1987; Rijsewijk et al., 1987). *Wg* function is required throughout development in a wide range of patterning events at different times and in different tissues. These include the development of embryonic epidermis, the head, the CNS, midgut, the heart, muscles and malpighian tubules (reviewed by Cadigan and Nusse, 1996). In imaginal discs, *wg* is expressed in a very complex pattern (Couso et al., 1993), and one interesting feature is that distinct enhancers control the different expression domains; enhancers that are themselves activated by different signalling pathways. In ventral discs (legs and antenna), *wg* is expressed in a sector that corresponds to ventral/anterior cells. The secreted protein Hedgehog activates the enhancer that drives this expression domain, which defines ventral fate and provides organising activity for the development of the proximodistal axis (Struhl and Basler, 1993; Basler and Struhl, 1994; Díaz-Benjumea et al., 1994). In the wing disc *wg* shows a very dynamic pattern of expression. In second instar larvae, *wg* is expressed in a ventral/anterior sector in a pattern similar to that displayed in

the leg. This early expression is required for the specification of wing fate (Morata and Lawrence, 1977) and is under the control of Hedgehog signalling pathway (J. D.-B., unpublished). Unlike in the leg, this expression later fades away and *wg* starts to be expressed in a wide stripe that corresponds to the presumptive wing margin. The Notch signalling pathway also controls this enhancer. In this way, *Wg* is involved in the specification of the wing margin, which is required for the promotion of cell proliferation and patterning of wing cells (Phillips and Whittle, 1993; Díaz-Benjumea and Cohen, 1995; Zecca et al., 1996; Neumann and Cohen, 1997). In the mesothorax, *wg* is expressed in an anterior/posterior stripe, and is required for the specification of dorsocentral bristles. The enhancer that positions this stripe seems to be controlled by the GATA protein Pannier and the zinc-finger protein U-shaped (García-García et al., 1999). *wg* is also expressed in two concentric rings that surround the wing pouch. The inner ring (IR) is activated in early third instar, and the outer ring (OR) is activated in late third instar. These two rings define the wing bases (also called wing hinge), which is the region that joins the wing and thorax. This region has a complex structure that is required for the fluttering of the wing. In some *wg* mutants, this region is deleted and the wing is joined directly to the thorax. The enhancer that drives *wg* expression in the IR has been identified within a 1.2 kb DNA fragment located about 9 kb 5' of the *wg* promoter (Neumann and Cohen, 1996). This fragment is sufficient to direct reporter gene expression in the IR. *wg* alleles that specifically affect this enhancer have been

characterised as *spade* mutants (Couso et al., 1994; Tjong and Nash, 1990).

We have analysed the mechanisms that drive *wg* expression in the IR. We present evidence that indicates that the genes *vestigial* (*vg*), *rotund* (*rn*) and *nubbin* (*nub*) are required, and that *wg* expression in the IR is driven by two independent mechanisms. The first initiates *wg* expression in early third instar larvae, and depends on cell interactions between *vg*-expressing and *vg*-non-expressing cells. The second mechanism is required for the maintenance of *wg* expression, and depends on an autoregulatory loop that requires the function of the genes *homothorax* (*hth*) and *rn*. *hth* expression in the IR seems to be controlled by Wg signalling, but *rn* expression depends on a signal secreted by the *vg*-expressing cells. Thus, *wg* expression in the IR is not maintained by lineage. We also present evidence that indicates that Wg function in the IR promotes the patterning of the hinge by generating different domains, which are defined by different combinations of gene expression.

MATERIALS AND METHODS

Fly strains

The following fly strains were used: *spd^{flg}* and *spd-lacZ* (Neumann and Cohen, 1996); *rn^{Δ2.2}*, *rn-lacZ*, *UAS-rn* and *rn-GAL4* (St Pierre et al., 2002); *nub¹* and *nub²* (Ng et al., 1995); *vg^{83b27R}* (Williams et al., 1993); *N^{ts}* (Shallenberger and Mohler, 1978); *hth-lacZ* (Rieckhof et al., 1997); *UAS-vg* and *vg^{QE}-lacZ* (Kim et al., 1996); *UAS-hth* (Casares and Mann, 1998); *dpp-GAL4* (Wilder and Perrimon, 1995); *ap-GAL4* (Calleja et al., 1996); and *en-GAL4* and *wg-lacZ* (Kassis et al., 1992).

Clonal analysis

To induce clones of ectopic expression, *y hs-FLP122; Ac>y⁺>GAL4 UAS-GFP* females were crossed either with *UAS-vg* or *UAS-rn* males. Embryos were collected after 24 hours and heat shocked at 34.5°C for 12 minutes at 36±12 hours of development.

To induce loss-of-function clones, embryos from the appropriate crosses were collected for 24 hours and heat shocked at 37°C for 1 hour at 36±12 hours of development. The genotype examined were: for *rn* clones, *y hs-FLP122; rn^{Δ2.2} FRT[80] / Ubi-GFP FRT[80]*; for *nub* clones, *y hs-FLP122; nub¹ FRT[40A] / Ubi-GFP FRT[40A]*; and for *hth* clones, *y hs-FLP122; hth^{P2} FRT[80] / Ubi-GFP FRT[80]*.

Lineage tracing

Ac>y⁺>GAL4 UAS-GFP / UAS-FLP; rn-GAL4 / rn-lacZ larvae were generated, dissected and stained with anti-β-galactosidase for analysis under the confocal microscope. This experiment lineage-tagged cells that expressed *rn* at any time during the development of the disc. FLP recombinase is expressed in cells expressing *rn-GAL4*, and mediates excision of the flip-out *y⁺* cassette from the inactive construct to generate an active *Ac>>GAL4* transgene that will express *UAS-GFP*. After excision of the cassette, *GAL4* expression is regulated by the actin promoter and is clonally inherited in all the progeny of *rn-GAL4*-expressing cells (Struhl and Basler, 1993; Weigmann and Cohen, 1999). The pattern of GFP expression is compared with the pattern of β-galactosidase expression that shows the final pattern of *rn* expression.

Immunostaining

Discs were dissected in PBS + 0.1% Tween and fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. Standard protocols for immunostaining were followed. The antibodies used were: mouse anti-Dll (Vachon et al., 1992); mouse anti-Nub (Ng et

al., 1995); rabbit anti-Vg (Williams et al., 1991); mouse anti-Wg (Brook and Cohen, 1996); and rabbit anti-β-galactosidase (Cappel).

RESULTS

The genes *wingless*, *rotund* and *nubbin* are required for the development of the wing hinge

The adult *Drosophila* wing is formed by a continuous monolayer of epidermal cells that folds to form the dorsal and ventral surfaces of the wing pouch. The two surfaces contact at the margin of the wing and extend proximally through the wing hinge to the dorsal notum and the ventral pleura. In the presumptive wing region of the wing disc, *wg* is expressed in a narrow stripe of cells that run all along the wing margin and in two rings that surround the wing pouch (Fig. 1A,B). We have examined the phenotypes and *wg* expression in several mutants in which the wing hinge is deleted.

The effects of removing *wg* expression in the IR can be observed in *spade* (*spd*) mutants (Tjong and Nash, 1990; Couso et al., 1994). *spd* mutations are a type of *wg* alleles that specifically removes *wg* expression from the IR, with no effects on other expression domains (Fig. 1C). In *spd^{flg}* wings, the hinge region is deleted, and the wing pouch appears directly joined to more proximal cells. In these wings, both *wg*-expressing cells and surrounding cells are missing. It has been shown that this phenotype is not caused by cell death is rather a consequence of underproliferation in this region, suggesting that one of the functions of Wg in the IR is to promote local cell proliferation (Neumann and Cohen, 1996).

The *rotund* (*rn*) gene is a member of the Krüppel family of zinc-finger encoding genes (St Pierre et al., 2002). Among other phenotypes, *rn* mutations delete the wing hinge and remove *wg* expression from the IR (Fig. 1D).

The *nubbin* (*nub*) gene encodes a member of the POU family of transcription factors (Ng et al., 1995). In strong *nub* mutations wings are vestigial, but phenotypic analysis of weaker alleles shows that the wing hinge is deleted and the expression of *wg* in the IR is missing (Fig. 1E). We examined the hinge phenotype of the triple mutant *spd^{flg} nub²; rn^{Δ2.2}* and it is similar to the phenotype of all of them, suggesting that the main cause of the phenotype is the lack of *wg* expression in the IR (data not shown).

vg, *rn* and *nub* genes are expressed in three concentric domains in the wing pouch

The gene *vestigial* (*vg*) encodes a nuclear protein with no homology with other identified families of nuclear proteins (Williams et al., 1991). Based on its interaction with scalloped (*sd*), a transcription factor with a TEA/ATTS DNA-binding domain, it has been suggested that the function of Vg is to mediate transcriptional activation by Sd (Paumard-Rigal et al., 1998; Simmonds et al., 1998). *vg* expression in the wing is regulated by two separate enhancers: the boundary enhancer (BE) and the quadrant enhancer (QE). The BE is activated by the Notch signalling pathway and drives *vg* expression at the dorsal/ventral boundary in middle/late second instar larval stage. The QE is activated by the combined action of Wg and Dpp (a TGFβ homologue), and drives *vg* expression in the rest of the wing pouch from early third instar larval stage (Williams et al., 1994; Kim et al., 1996; Neumann and Cohen, 1997).

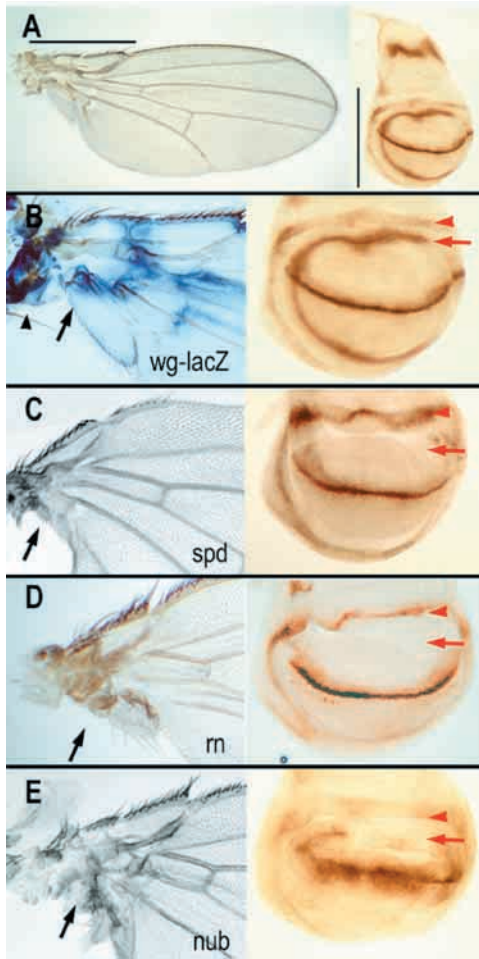


Fig. 1. *wg* expression and phenotypes of wild-type and mutant wing hinges. (A) Adult wing and third instar larval wing imaginal disc showing *wg* expression detected by antibody staining. Bars indicate the regions amplified in B–E. (B) *wg* expression detected by X-gal staining in adult wing and by anti-Wg antibody in the wing pouch. Red arrows indicate the inner ring (IR); black arrows indicate the corresponding region in the adult wing. The arrowhead indicates the outer ring (OR). (C–E) Mutant phenotypes of *spd^{lg}* (C), *rn^{Δ2-2}* (D) and *nub²* (E). In all cases the hinge is deleted and the expression of *wg*, which is detected by staining of antibody to Wg, in the IR is missing.

We examined the expression patterns of *vg*, *rn* and *nub*. In mature wing discs *vg*, *rn* and *nub* are expressed in three concentric domains, with the Vg domain the smallest one. At this stage the wing hinge is lined with several anterior/posterior folds (Fig. 2K). The boundary of *vg* expression coincides with the distal-most fold of the disc (Fig. 2E,K). The Rn domain is slightly broader and its boundary coincides with a second fold in the disc (Fig. 2F,K). The Nub domain contains the Rn domain and coincides with the third fold in the disc (Fig. 2G,K). The IR domain corresponds to the proximal-most area of the Rn domain (Fig. 2F inset, K).

We next examined the expression of these genes in early larval development. In middle/late second instar larvae the expression domains of *vg*, *rn* and *nub* in the presumptive wing pouch are slightly broader than the *vg* domain (Fig. 2A,B). The

rest of the cells of the disc, which do not express *nub*, express the gene *teashirt* (*tsh*) (Ng et al., 1996; Fasano et al., 1991). *wg* is expressed only in a stripe of cells that corresponds to the presumptive wing margin (Fig. 2C). In early third instar larvae, *wg* starts to be expressed in the IR (Fig. 2D). This expression domain corresponds to cells that express *rn* and *nub* but do not express *vg*. *wg* expression in the IR promotes the growth of the hinge (Neumann and Cohen, 1996) and, in third instar larvae, gives rise to the expression patterns described above for *vg*, *rn* and *nub*. At this stage, the cells that express the IR enhancer are located at the limit of the domain 3 (Rn + Nub), and are several cells away from the boundary of *vg* expression (Fig. 2E). To investigate how the IR enhancer would be expressed in *spd^{lg}* discs, we examined *lacZ* expression in transgenic flies carrying *lacZ* driven by the DNA fragment that contain the IR enhancer (Neumann and Cohen, 1996). In wild-type discs the *spd-lacZ* construct is activated in a ring around the wing pouch that corresponds to the IR but also in the wing margin (Neumann and Cohen, 1996). In *spd^{lg}* discs of third instar larvae the *spd-lacZ* expression remains adjacent to the Vg domain as a consequence of local underproliferation, and the Rn and Nub domains, which contain the cells that express the *spd-lacZ*, are only slightly broader than the Vg domain (Fig. 2H–J).

Expression of *wg*, *rn* and *nub* in the wing pouch requires Vg

As *vg*, *rn* and *nub* are expressed in the wing pouch in very similar domains, we wanted to determine whether they could be placed in a regulatory cascade. *vg^{83b27r}* is considered a null allele and produces flies with no wings (Williams et al., 1993). We observed that in *vg^{83b27r}* wing discs expression of *wg*, *rn* and *nub* was not detected in the wing pouch, although other domains of expression in notum or legs were not affected (Fig. 3A–C). We observed the same result in earlier discs, suggesting that Vg is required to initiate the expression of *wg*, *rn* and *nub* in the wing pouch.

To confirm these results and to test whether *vg* expression is sufficient to activate *wg*, *rn* and *nub*, we made use of the *UAS/GAL4* system (Brand and Perrimon, 1993) to ectopically express *vg*. *vg* misexpression in the notum does not activate neither *rn* nor *nub*, so we tested other discs. In the eye discs of wild-type larvae, Rn and Nub are not expressed, and in the antenna discs they are expressed in the same pattern as in the leg, with Rn in a broad ring and Nub in several narrow rings (Fig. 3B,C). In *dpp-GAL4/UAS-vg* eye/antenna discs, Rn and Nub are detected both in the eyes and in the antennae in the Dpp domain (Fig. 3E,F).

We also examined the expression of Vg and Rn in *nub¹* discs and the expression of Vg and Nub in *rn^{Δ2-2}* discs. In both cases we did not detect changes in the expression patterns of these genes (data not shown). Ectopic expression of either *rn* or *nub* did not drive the expression of the other gene (data not shown). From these results, we conclude that Vg is necessary and sufficient to activate the expression of *rn* and *nub* in the wing pouch.

Although, in late second instar larvae Vg, Rn and Nub are found in almost coincident domains, in third instar the Rn and Nub domains include two sectors that do not express *vg* (Fig. 2K, domains 2 and 3). This observation suggests either that the activation of *rn* and *nub* is mediated by a non-autonomous

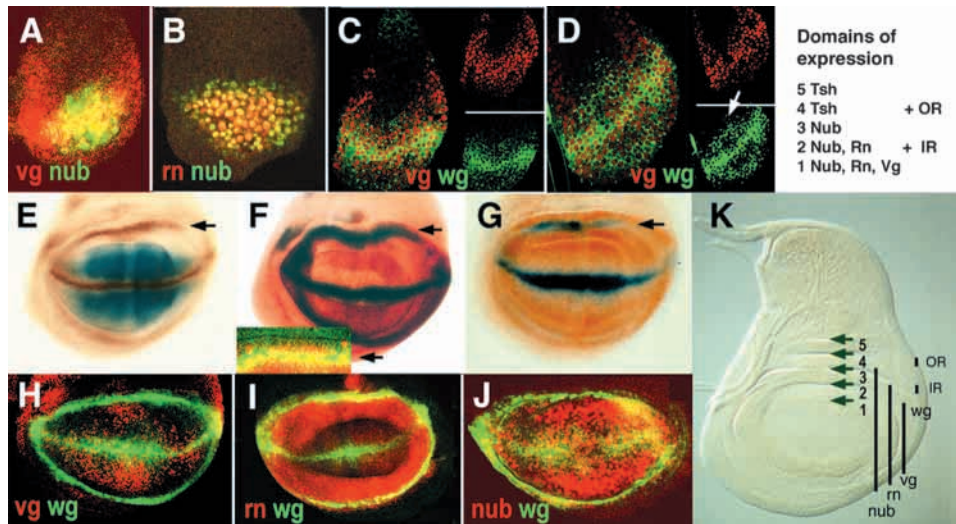


Fig. 2. *vg*, *rn* and *nub* expression patterns and their relationship to the IR. Wild-type wing disc from late second (A–C), early third (D) and late third (E–G) instar larvae. (H–J) *spd-lacZ* mature wing discs. In late second instar larvae *vg*, *rn* and *nub* expression domains are almost coincident (A–B), and *wg* is expressed in a stripe that delimits the presumptive wing margin (C). In early third instar larvae, *wg* is also expressed in a ring of cells that do not express *vg* (D, white arrow). (E–G) In mature wing discs *vg*, *rn* and *nub* are expressed in three concentric domains (arrows): *vg* and *wg* (E), *rn* and *wg* (F), and *nub* and *wg* (G). (H–J) *spd-lacZ*, *vg*, *rn* and *nub* expression patterns in *spd-lacZ* discs. Note that the IR co-expresses with *rn* (I) and *nub* (J), but abuts the *vg* domain (H). Because of different focal planes it is not possible to merge the different channels over the whole of the Wg domain at this magnification. (K) Summary of patterns of gene expression. The mature wing hinge has five characteristic folds (arrows) that coincide with domains of gene expression. Vertical bars represent the domains of expression of *nub*, *rn*, *vg* and *wg* in the IR and the OR. Note that although domain 2 includes the IR, only the cells in the proximal-most sector express *wg*. In all discs, anterior is leftwards and the dorsal notum is upwards. The wing margin, identified by *wg* expression, is considered the distal-most region of the wing.

mechanism, or that although Vg is required to initiate *rn* and *nub* expression, Vg-independent mechanisms might be required to maintain them (see below).

Requirement for the activation of *wg* in the inner ring

In *vg^{83b27R}* larvae, the IR is missing, but *vg* misexpression in the eye/antenna disc is not able to activate *wg* expression. This suggests that a more sophisticated mechanism may drive the expression of *wg* in the IR. *vg* is not expressed in the hinge. When *vg* is misexpressed in the IR domain by any one of several different GAL4 lines, *wg* expression is lost (Fig. 4I and data not shown). This indicates that Vg, although required for the IR activation, represses the IR when both are co-expressed in the same cells.

To better assess the role of Vg in the activation of *wg* expression in the IR, we made clones of *vg*-expressing cells in the different expression domains of the wing hinge (Fig. 2K). These clones behave differently depending on the domain where they appear. In domains 2 (Nub + Rn) and 4 (Tsh), *vg*-expressing clones did not induce *wg* expression. In domain 3 (Nub), clones of *vg*-expressing cells produce a non-autonomous expression of *wg*. In these clones, induced at 36±12 hours of development, we found three distinct results: first, *wg* is expressed in a line of cells surrounding the clone (Fig. 4A); second, the domain of cells expressing *wg* becomes broader (Fig. 4B); and third, *wg*-expressing cells form a ring

that lies several cells away from the boundary of the clone (Fig. 4C). We conclude that the behaviour of these clones and their surrounding cells reproduces the normal development of *wg* expression in the IR. *wg* expression is first activated in cells abutting the Vg domain, and this ring of expression later moves away from the Vg domain. We propose that a signal from the *vg*-expressing cells induces *wg* expression in surrounding cells. As a consequence of Wg-promoted cell proliferation, the IR moves several cells away from the boundary of *vg*-expressing cells. Because in *vg*-expressing clones the cells that express *vg* are related by lineage, we must assume that some cells lose the expression of Wg and that this happens at least in the internal border of the IR (see Discussion).

To assess the role of Rn in this signalling, we examined the expression of *rn* in these *vg*-expressing clones. Clones of *vg* expression in domain 3 (Nub) activate *rn* expression. This expression is not limited to the cells of the clone, but the surrounding cells also express *rn* (Fig. 4D,E). Considering that Vg is a nuclear protein, a Vg-dependent signalling

may be required to activate *rn* expression. The activation of *rn* in these clones seems to be restricted to domain 3 (Nub). One explanation could be that Nub is required. Nevertheless, *rn* expression was not altered in *nub¹* discs, in which Nub protein is detected by antibody staining in only a few cells (Ng et al., 1995; Cifuentes and García-Bellido, 1997). We therefore think that the activation of *rn* by Vg-dependent signalling may be repressed in *tsh*-expressing cells (domain 4). Similar to the observed dynamics of *wg* expression, we found clones in which *rn* is only expressed within the cells of the clone, and clones in which *rn* is also expressed in the surrounding cells. It is interesting to note that only clones in which *rn* expression is non-autonomously activated show *wg* expression (Fig. 4F). These results suggest that in early third instar larvae, a Vg-dependent signalling pathway non-autonomously activates *rn* expression in neighbouring *vg*-non-expressing cells, and this makes these cells competent to activate the IR enhancer.

To determine whether Rn is by itself able to activate *wg* expression in cells that do not express *vg*, we examined the expression of *wg* in clones of *rn*-expressing cells. These clones, when induced in domain 3 (Nub), activate *wg* expression. Unlike *vg*-expressing clones, in these clones *wg* expression is restricted to cells of the clone (Fig. 4G,H). In addition, within the clones the only cells that express *wg* are those lying close to the Vg domain. This suggests that Rn, although required for *wg* expression, it is not sufficient. We think that when the IR

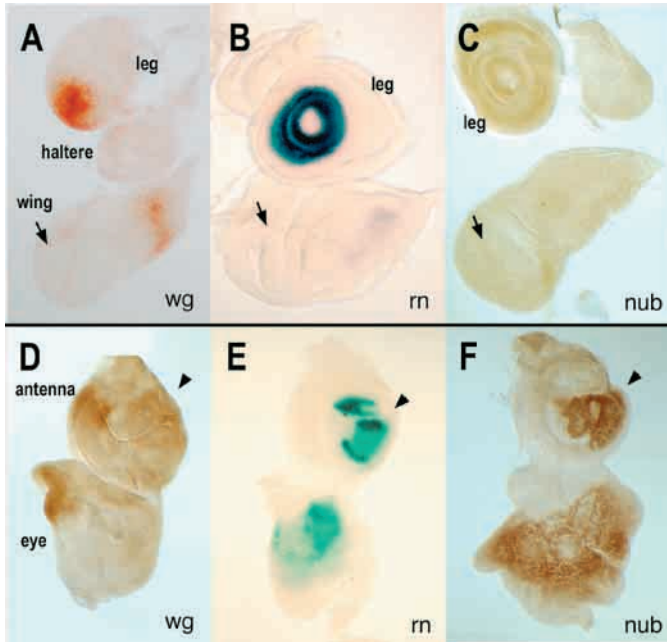


Fig. 3. *vg* is required to activate the expression of *wg*, *rn* and *nub* in the wing pouch. (A–C) *vg*^{83b27R}. The expression of *wg* (A), *rn* (B) and *nub* (C) in the wing pouch is missing (arrows; see Fig. 1F,G for wild-type expression). The expression in notum and legs was not affected. Ectopic expression of *vg* is sufficient to activate *rn* and *nub*. (D–F) *dpp-GAL4/UAS-vg*. Patterns of expression of *wg* (D), *rn* (E) and *nub* (F) in the eye/antenna disc. Arrowheads indicate the *dpp* expression domain in the antenna. *rn* and *nub* are expressed in similar patterns in leg and antenna. In D–F, ventral is leftwards.

was activated, some of these clones had a few cells, but only the cells that lie close to the Vg domain activated *wg*. After several cell divisions, some of these cells lie out of the IR domain but, because they express *rn*, they retain the expression of *wg*.

In view of these results, we propose a two-step model, in which Vg first non-autonomously activates *rn* expression both within the Vg domain and in surrounding cells. This generates two adjacent domains: *vg/rn*-expressing cells and *rn*-expressing cells. Cell interactions between these two groups of cells then drive the activation of the IR enhancer in *rn*-expressing cells.

Rn and Nub are cell autonomously required for the activation the *wg* expression in the IR

The results presented above indicate that Rn and Nub are required for the activation of the IR and that this activation depends on a signal from *vg*-expressing cells. To identify in which cells Rn and Nub functions are required for IR activation, we performed genetic mosaic analysis of strong loss-of-function alleles of *rn* and *nub*.

rn^{Δ2-2} is considered to be a null allele (St Pierre et al., 2002). *rn*^{Δ2-2} homozygous flies are viable, and display as wing phenotypes the deletion of the wing hinge and a nick in the posterior proximal wing margin (St Pierre et al., 2002). Clones of *rn*^{Δ2-2} cells in a *rn*^{Δ2-2/+} background remove *wg* expression from the IR. This phenotype is strictly cell autonomous, indicating that Rn function is only required in the IR cells (Fig.

5A,B). *rn* clones that straddle the wing margin do not affect the expression of *wg*.

*nub*¹ is a viable and recessive strong loss-of-function allele that causes a dramatic reduction in wing size and the deletion of wing hinge (Fig. 1E) (Ng et al., 1995). We induced clones of *nub*¹ cells in an otherwise wild-type *nub*^{1/+} background. These clones remove *wg* expression. The phenotype is strictly cell autonomous, indicating that Nub is only required in the IR expressing cells (Fig. 5C,D). It therefore appears that Rn and Nub, although expressed in *vg*-expressing cells, are not required in these cells.

wg expression in the inner ring requires cell interactions between *vg*-expressing and *vg*-non-expressing cells

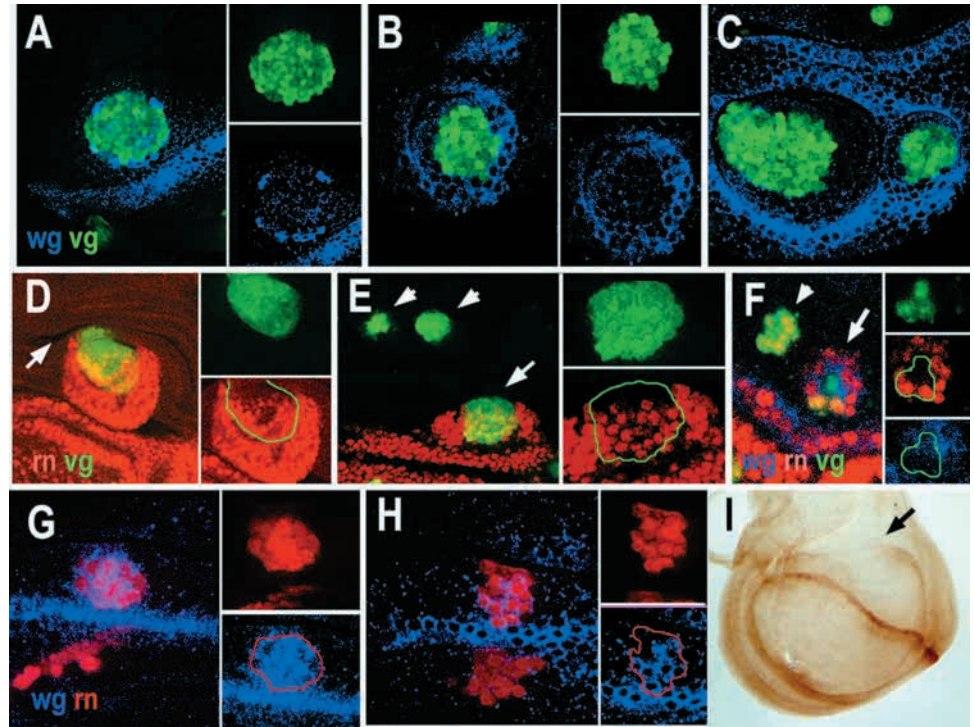
As we have seen above, *vg* expression in second instar larvae drives the expression of *rn* and *nub*, two genes that are required both for the development of the wing hinge and for the activation of the *wg* IR enhancer. Clones of *vg*-expressing cells in the Nub domain activate the IR enhancer. These ectopic IR domains, although generated in cells that abut the clone boundary, end up several cells away. This behaviour reproduces the normal development of the IR, which is initially expressed at the boundary of *vg* expression, but later, as a result of Wg-induced cell proliferation, moves several cells away. These observations suggest that a signal coming from *vg*-expressing cells activates the IR enhancer in the surrounding cells.

To test this hypothesis, we generated a new artificial boundary of *vg* expression at an ectopic position, by removing *vg* expression in cells within the *vg* domain. *vg* mutant clones do not generate this situation, as they do not proliferate for more than a few cell divisions (Azpiazu and Morata, 2000). We therefore misexpressed the gene *homothorax* (*hth*) in the Vg domain. *hth* encodes a homeodomain protein of the Meis family (Rieckhof et al., 1997). In the wing, *hth* is expressed in two rings that overlap with the IR and the OR of Wg (Casares and Mann, 2000; Azpiazu and Morata, 2000), and it has been suggested that Hth interferes with Wg signalling (Abu-Shaar and Mann, 1998).

In *dpp-GAL4/UAS-hth*, the Wg-dependent *vg* expression was repressed in the Dpp domain (Fig. 6A, note that the N-dependent BE is not affected, indicating that Hth does not interfere with N signalling). In these discs, *rn* and *nub* expression are not affected (Fig. 6B,C), and two new stripes of *wg* expression appear (Fig. 6D–F, note that not all cells that express *hth* activate *wg* expression). These two new stripes of Wg are clearly seen in middle third instar larvae, and correspond to Dpp-expressing cells that abut the *vg* expression domain. In mature larvae the epithelia is folded, and only a stripe of Wg is detected (Fig. 6F).

To confirm that this ectopic expression of *wg* is driven by the IR enhancer, we examined *Distal-less* (*Dll*) expression and N function. *Dll* is normally expressed in the wing pouch but is not expressed in the IR. Its expression depends of *wg* expression in the wing margin (Neumann and Cohen, 1997). In *dpp-GAL4/UAS-hth*, *Dll* expression is missing in the Dpp domain, where Wg is being ectopically expressed (Fig. 6G). *N* is required for the expression of *wg* in the wing margin but is not required for the activation of the IR enhancer. *N*^{ts} is a thermosensitive allele which at a restrictive temperature (30°C)

Fig. 4. *vg* and *rn* mediate the activation of the IR. (A–F) Clones of *vg*-expressing cells. Clone cells are labelled green (GFP), Wg is labelled in blue (wg antibody) and Rn in red (*rn-lacZ*). When we examine the expression of *wg* in these clones we found three distinct results: (1) *wg* is expressed in a narrow ring of cells that abuts the clone border (A); (2) *wg* is expressed in a broader ring of cells that still abuts the clone border (B); and (3) *wg* is expressed in a ring that stands several cells away from the clone border (C). We infer that these three distinct results represent three stages and reproduce the process of activation of the IR throughout normal development. (D–F) *vg*-expressing clones activate *rn* expression within the cells of the clone and in surrounding cells. This activation is restricted to the Nub domain. (D) Non-autonomous activation of *rn* in a *vg*-expressing clone. The arrow indicates the fold that delimits the expression of *nub*. (E) Two clones out of the Nub domain (arrowheads) and a clone within the Nub domain (arrow). Owing to different planes of focus, not all the cells of the clone show *rn* expression. (F) *vg*-expressing clones that do not activate *rn* expression in cells out of the clone do not express *wg* (arrowhead). (G,H) Clones of *rn*-expressing cells: clone cells are red (GFP) and Wg is blue. Rn autonomously activates *wg* expression, but only in the Nub domain and in cells that are close to the Vg domain. (I) *wg* expression in *ap-GAL4/UAS-vg* wing disc. Note the lack of dorsal *wg* expression (arrow).



behaves as a strong loss-of-function allele. In *N^{ts}; dpp-GAL4/UAS-hth*, the expression of *wg* in the wing margin is missing, but neither the ectopic *wg* expression in the Dpp domain nor the *wg* expression in the IR is affected (Fig. 6H). This result suggests that both domains are independent of N signalling.

For final confirmation that cell interactions between *vg*-expressing and *vg*-non-expressing cells activate the IR enhancer, we tested whether *hth* misexpression was able to activate *wg* directly by examining *wg* expression in *engrailed-GAL4/UAS-hth*. In these discs, *hth* is expressed in the whole posterior compartment, but Wg is only detected in posterior cells that abut *vg*-expressing cells in the anterior compartment (Fig. 6I).

Hth mediates wg autoregulation in the IR

Once the IR enhancer is activated, Wg-induced local cell proliferation moves the IR several cells away from the boundary of *vg* expression. This cell proliferation generates three new domains defined by different combinations of gene expression. These are: cells expressing Nub+Rn; cells expressing Nub+Rn+IR; and cells expressing Nub (Fig. 2K). This raises the question of how *wg* expression is maintained far from the Vg boundary. To address this we examined the role of *hth*.

In second instar larvae, *hth* is expressed in the wing disc at low levels, in a pattern complementary to *vg*. In early third instar larvae, after *wg* is expressed in the IR, *hth* starts to be expressed at higher levels in two rings that overlap with the IR and the OR of *wg* expression (Fig. 7A). Based on experiments

in which Wg signalling was compromised, it has been proposed that *hth* is a target of Wg signalling in the hinge (Casares and Mann, 2000). *hth* expression is missing in the *spdf^{ts}* mutant (Fig. 7B). Clones of *hth* mutant cells prevent *wg* expression at the late third instar stage (Fig. 7D) (Casares and Mann, 2000). But *hth* clones do not block *wg* expression observed in early third instar larvae (Fig. 7E). These observations suggest that *wg* expression, although induced by a Vg-dependent signal, is maintained by a different mechanism that requires Hth.

Although *wg*-expressing cells in the IR move several cells away from the Vg domain during the growth of the hinge, the proximal limit of the IR always coincides with the border of *rn* expression. Clones of *rn*-expressing cells in domain 3 (Nub) maintain *wg* expression in the proximity of the IR (Fig. 4G,H). Taken together, these results suggest that Rn is also required to maintain *wg* expression. In this model, *wg* would maintain its own expression by an autoregulatory loop that requires Hth and Rn. *hth* expression depends on Wg, but *rn* expression depends on a signal from *vg*-expressing cells. When IR cells proliferate and drop out of range of Vg-dependent signals, they would lose *rn* expression and, as a consequence of this, *wg* and *hth* expression may also be lost. Thus, *wg* expression, although maintained by an autoregulatory loop, would not be maintained by lineage alone.

To confirm this model, we conducted a lineage-tracing experiment to examine if cells at the border of the Rn domain have a tendency to lose *rn* expression. We compared the pattern of *rn* expression in mature discs with the distribution of cells that express *rn* at any time during larval development (see

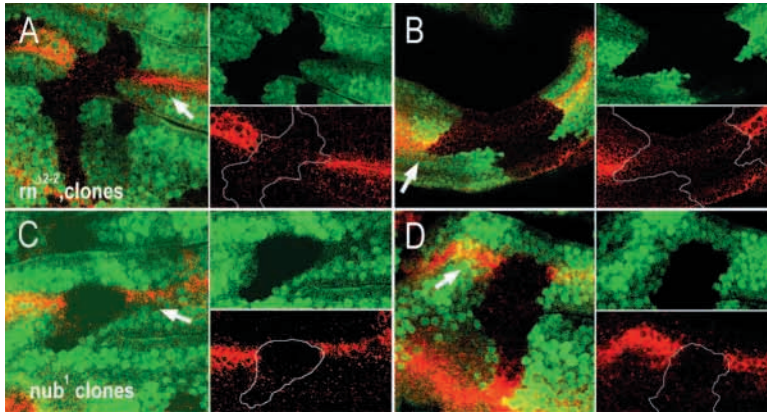


Fig. 5. Rn and Nub are required for *wg* expression in the IR. (A–B) *rn*^{Δ2-2} clones, revealed by the absence of GFP (green), cell autonomously remove *wg* expression (red) in the IR. Other domains of *wg* expressions are not affected. (C,D) *nub*¹ clones, revealed by the absence of GFP (green), also remove *wg* expression (red) in the IR. As it has previously been reported, *wg* expression in the wing margin is expanded in these clones (Neumann and Cohen, 1998). Arrows indicate the IR. *wg* expression is detected with antibody to Wg. Dorsal is upwards and anterior is leftwards.

Materials and Methods for details). When compared, we observed that the final domain of *rn* expression was smaller than the domain of cells that expressed *rn* in larval stages (Fig. 7C). This indicates that in development cells are dropping out of the Rn domain.

Interestingly, *wg* expression in the IR plays a role in maintaining *vg* expression. The sharp border of the Vg domain becomes less well defined in *spd*^{fs} wing discs, but is not affected in *N*^{ts} (30°) disc, in which both *vg* BE and *wg* expression from the wing margin have been removed (Fig. 7F–H). This implies that Wg from the IR helps to maintain the expression of the *vg* quadrant enhancer.

DISCUSSION

The very precise spatial and temporal control of gene expression plays an important role in pattern formation. One of the best-characterised examples of this is the development of the *Drosophila* embryo, in which sets of genes can be placed in a regulatory hierarchy, and have a very precise expression that is spatially and temporally regulated. In several cases, these genes are expressed at different times under the control of different enhancers, which in turn are activated by different developmental mechanisms (Pankratz and Jäckle, 1993). This precise control of gene expression is not restricted to early stages of development, and to some extent post-embryonic development introduces increased complexity, as pattern formation is associated with the control of cell proliferation (Day and Lawrence, 2000).

The *wg* gene has been exhaustively studied, and it has been reported that Wg plays multiple roles in the development of imaginal discs. In many cases, Wg has an instructive function, as its very precisely regulated expression is required for normal development (Klingensmith and Nusse, 1994; Martínez-Arias et al., 1999). One interesting feature is that an equally complex regulatory region controls the complex pattern of *wg* expression in the development of imaginal discs (Neumann and Cohen, 1996) (K. Johnson and J. P. C., unpublished).

wg is expressed in the wing in two rings that are required for patterning the wing hinge. This flexible region is required for wing flapping and for the movement of extension and flexion over the abdomen at rest (Snodgrass, 1935). We have examined the mechanisms that drive *wg* expression in the IR of the hinge. Our results indicate the expression of *wg* is

regulated by two different mechanisms, one to initiate and the other to maintain its expression. These processes involve the genes *vg*, *rn*, *nub* and *hth*, and other as yet unidentified genes.

A Vg-dependent signal activates *wg* expression in the IR

At middle second instar larvae, *vg* gene, as detected by Vg antibody staining, is expressed in the wing disc in a horseshoe-like domain. The centre of this domain corresponds to the presumptive wing pouch (Klein and Martínez-Arias, 1999; Williams et al., 1991). The results presented here indicate that Vg is required to activate the expression of *rn* and *nub* genes

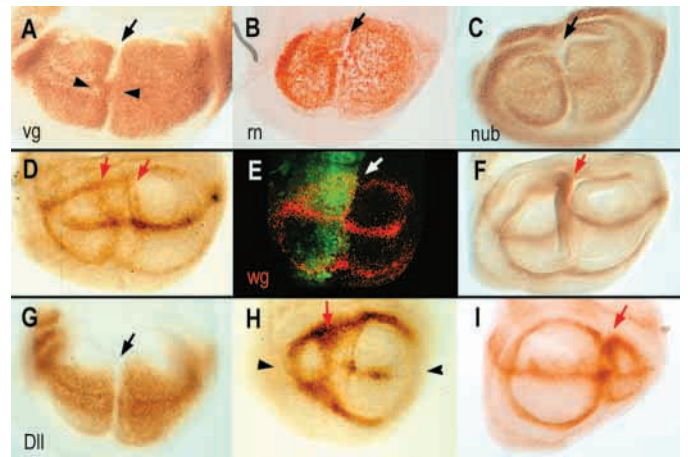
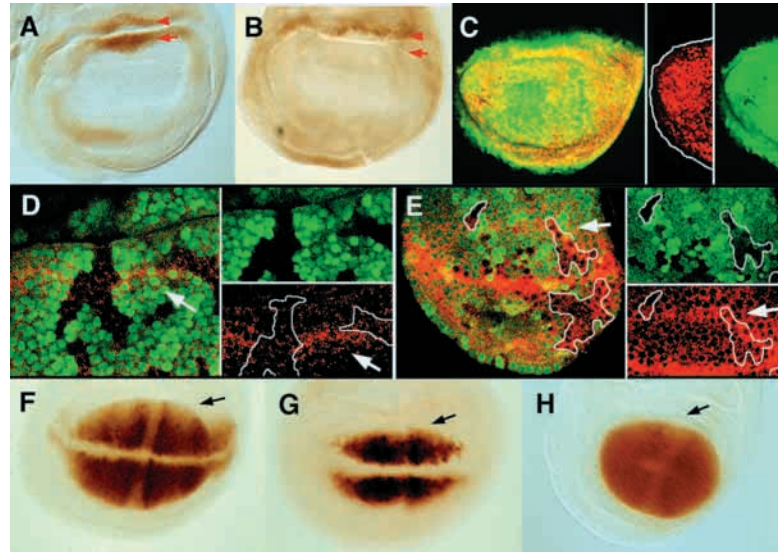


Fig. 6. Cell interactions at the boundary of *vg*-expressing cells drive the expression of the IR enhancer. (A–G) *dpp-GAL4/UAS-hth* removes *vg* expression (A), but does not affect expression of *rn* (B) or *nub* (C) (arrows). Note that the activation of the N-dependent *vg* BE is not repressed (arrowheads in A). *wg* is ectopically expressed within the Dpp domain in two stripes of cells that abut *vg*-expressing cells (D–F, arrows). In second instar larvae, the two stripes of Wg are more apparent (D). In third instar larvae, only one stripe is seen because of a fold in the epithelium (F). (G,H) Two results indicate that the new Wg stripe corresponds to the activation of the IR enhancer: *Dll* expression is missing (G); and *n*^{N^{ts}}; *dpp-GAL4/UAS-hth* (H), *wg* expression in the wing margin is missing (arrowheads) but the IR and the new stripe of *wg* is not affected (red arrow). (I) *hth* is unable to directly activate *wg* expression: in *en-GAL4/UAS-hth* only posterior cells in the AP boundary activate the IR (red arrow), indicating that cell interactions with *vg*-expressing cells are required.

Fig. 7. (A,B) *hth* expression detected with antibody to β -galactosidase in *hth-lacZ* wing discs. *hth* is expressed in the wing hinge in two rings (A). The inner ring (red arrow) and the outer ring (red arrowhead) overlap with the IR and the OR of Wg. (B) In *spd^{lg}* discs, the *hth* expression in the inner ring is missing but the outer ring is not affected. (C) *rn* lineage tracing. Expression of *rn* visualised by *rn-lacZ* (red) and by *rn-GAL4/UAS-FLP Act>>GAL4/UAS-GFP* (green), which labels all the cells that expressed *rn* at any time during the development of the disc. Note that the green channel shows a broader domain, indicating cells that have lost *rn* expression. This result suggests that in the border of Rn domain, cells have a tendency to lose *rn* expression. (D,E) *hth^{P2}* clones, revealed by the absence of GFP (green), remove *wg* expression (red) in the IR (white arrows) when observed in mature wing discs (D), but do not do so when observed in earlier discs (E). This indicates that the maintenance, but not the initiation, of *wg* expression requires Hth function. (F-H) *wg* expression in the IR is required to maintain sharp borders in the *vg* expression domain. Expression of *vg QE-lacZ* in wild-type (F), *spd^{lg}* (G) and *N^{ts}* (H) [at restrictive temperature (30°C)] wing discs detected with antibody to β -galactosidase. The border of the Vg domain (black arrows) is sharp in wild-type larvae or when *wg* is removed from the wing margin (H), but less well defined when *wg* is removed from the IR (G).



in the wing disc. This activation is restricted to the cells that will take wing fate and takes place in the cell that express *vg*, and also in the surrounding cells, suggesting that a Vg-dependent short-range signal activates *rn* and *nub* expression. At this time, the expression of *nub* and *tsh* in the wing disc are complementary and cover the whole disc (Ng et al., 1996) (Fig. 8).

The expression of these genes in a domain broader than the Vg domain creates a ring of cells that express *rn* and *nub* but not *vg*. We have presented evidence indicating that a signal from *vg*-expressing cells activates the *wg* IR enhancer in adjacent *rn/nub*-expressing cells. Unlike the activation of *rn* and *nub*, the activation of *wg* expression by the IR enhancer is repressed in cells that also express *vg*. So, the IR enhancer is activated only in cells that surround the Vg domain.

During the development of the disc, the position of the IR moves several cells away from the Vg domain. This implies

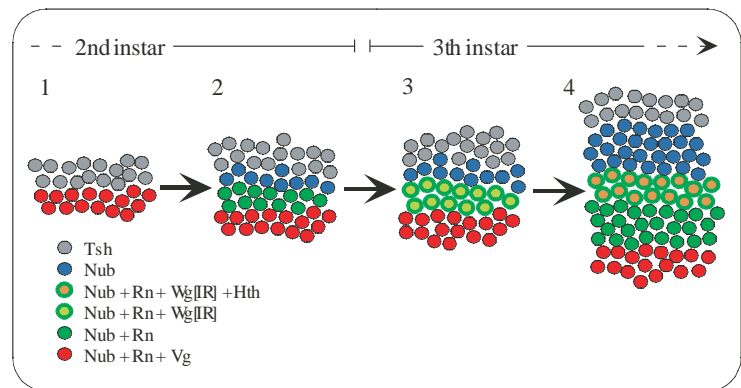
either that the Vg-dependent signalling is able to activate the IR over a long range (Liu et al., 2000), or that a different, Vg-independent, mechanism maintains the IR.

Hth mediates the maintenance of *wg* expression in the IR

When artificial Vg/Rn-Nub interfaces are generated experimentally, the IR enhancer is activated in *rn-nub*-expressing cells that abut the Vg domain (Fig. 6D-F). This ectopic IR is around four cells wide, indicating the active range of the signal that activates *wg* expression. Our results indicate that at distances greater than this, a Vg-independent mechanism maintains *wg* expression in the IR.

Several results presented here, plus others reported elsewhere, indicate that Wg signalling activates *hth* expression, which is in turn required to maintain *wg* expression. *wg* and *hth* are co-expressed in the IR and OR, and *wg* expression

Fig. 8. A model for the development of the wing hinge. The figure represents the evolution of gene expression during development in cells of the dorsal wing hinge. In second instar larvae *vg* (red) and *tsh* (grey) are expressed in complementary patterns (1). The *vg*-expressing cells correspond to the presumptive wing pouch. A signal coming from the *vg*-expressing cells activates *nub* and *rn* expression in slightly broader domains (2). In early third instar larvae, cells expressing *rn* and *nub* but not *vg* (green) are competent to activate the *wg* IR enhancer (yellow) when induced by a signal coming from the *vg*-expressing cells (3). *wg* expression stimulates local cell proliferation that expands the different domains and moves the IR domain several cells away from *vg*-expressing cells (4). At this time, Wg signalling activates *hth* expression in the IR (orange). The combination of both Wg-dependent Hth and Vg-dependent Rn activates a Vg-independent mechanism that maintains *wg* expression in the IR. As soon as local cell proliferation moves *rn*-expressing cells away from the Vg domain, they lose *rn* expression, and consequently also lose *wg* and *hth* expression. Thus, *wg* expression in the IR is maintained at the border of the Rn domain, which proximally restricts the IR domain. In this process three new domains have been generated by local cell interactions that do not involve any cell lineage restriction. Note that the cells that belong to the new domain of *rn*-expressing cells between the IR and the Vg domain (green cells in 4) loses the ability to activate *wg* by a mechanism that, we propose, involves a Vg-dependent repressor. Therefore, the IR domain is proximally and distally restricted.



precedes *hth* expression. Furthermore, *hth* expression is missing in *spd⁸* discs, and *wg* expression is lost in *hth* mutant clones. Nevertheless, *spd⁸* discs show activation of the IR enhancer, as revealed by the *spd-lacZ* construct (Fig. 2H-J) and *wg* expression is not affected in *hth* mutant clones when observed in early third instar larvae (Fig. 7E). This indicates that Hth, while required to maintain IR activation, is not required to initiate *wg* expression.

The *rn* clonal analysis indicates that Rn is also required for *wg* expression. One interesting observation is that, when the IR moves away from the Vg domain, *wg*-expressing cells are always maintained at the limit of *rn* expression. *rn* is activated by a Vg-dependent signal. This implies that the activity range of the signal and the lifetime of the Rn protein together limit the domain of *rn* expression. So one explanation for why the IR is always maintained in the limit of *rn* expression is that, as a consequence of cell proliferation, cells drop out of the range of the Vg-dependent signalling. Thus, cells simultaneously lose the expression of both *rn* and *wg*. The result of the *rn* lineage-tracing experiment supports this prediction (Fig. 7E). Taken together, these results suggest that an autoregulatory loop involving Hth and Rn maintains *wg* expression. Although *hth* expression depends on Wg, *rn* expression depends on Vg, so *wg* expression in the IR is not maintained by lineage. *wg* autoregulation has been reported in embryo development (Hooper, 1994; Manoukian et al., 1995), and a negative mechanism of 'self-refinement' has been suggested in wing margin specification (Rulifson et al., 1996). However, in neither of these cases has a role been reported for Hth or Rn.

Vg-dependent signalling activates four different target genes

Wg-promoted cell proliferation generates a new domain between the IR and the Vg domain (Fig. 2K, domain 2). This indicates that at this stage Vg-dependent signalling is unable to activate *wg* expression in adjacent cells. Otherwise the IR would be expressed in the whole Rn domain. One explanation for this could be that there is a temporal window for the activation of *wg*, but *vg*-expressing clones induced in mid/late third instar larvae are able to activate *wg* (data not shown). Another explanation could be that a repressor is expressed in this domain (Liu et al., 2000). Clones of *vg*-expressing cells placed in this domain do not activate *wg*, which supports this explanation. In the experiment in which we prevented *vg* expression in the Dpp domain, the new stripe of ectopic Wg did not recognise this domain (Fig. 6D-F), suggesting that the proposed repressor may be a target of Vg signalling. One alternative explanation is that *wg* refines its own expression domain by repressing the Vg-dependent activation. This has been proposed for the expression in the wing margin (Rulifson et al., 1996), but does not seem to be the case here. In experiments in which third instar larvae carrying a thermosensitive allele of *wg* (*wg^{ts}/wg^{cx4}*) were reared at the restrictive temperature (16 hours at 30°C) and stained with Wg antibody, we did not detect changes in the pattern of *wg* expression in the IR (data not shown). However, we observed that the expression in the wing margin was widened (Rulifson et al., 1996).

Thus, the proximal and distal limits of the IR would be defined respectively by the limit of *rn* expression and by the limit of the expression of the proposed repressor. In summary,

our results suggest that at least four different target genes are independently activated by one or more signals that emanate from *vg*-expressing cells: *rn* and *nub* are activated in second instar larvae; *wg* is activated in early third instar larvae (this activation requires the function of Rn and Nub and is repressed by Vg); and finally the repressor, which would be activated in middle third instar larvae.

The role of Wg in patterning the hinge

One interesting observation that can be made our results relates to how the hinge is patterned. As a result both of local cell interactions and Wg-promoted cell proliferation, several domains, which are defined by different combinations of gene expression, are established (Fig. 8). The generation of these domains is, in part, a consequence of that the expression of these genes are not maintained by lineage, but also because there is not evidence of lineage restrictions. Thus, cells at the borders of both the IR domain and the Vg domain lose *wg* and *vg* expression, and fall into adjacent domains. However the expression in cells within a given domain, away from the border, must be more efficiently maintained by a phenomena similar to the reported community effect (Gurdon et al., 1993), because no holes are detected in the pattern of expression.

We thank Natalia Azpiazu, Sean Carroll, Steve Cohen, Richard Mann and the Bloomington Stock Centre for fly stocks. We are grateful to Sean Carroll for the Vg antibody, to Steve Cohen for Dll, Wg and Nub antibodies, and to Sonsoles Campuzano for critical reading of the manuscript. This work has been supported by a grant from the Ministerio de Educación y Cultura (PM97-0145) and an institutional grant from the Fundación Ramón Areces to the Centro de Biología Molecular-Severo Ochoa.

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