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Genetic requirements of *vestigial* in the regulation of *Drosophila* wing development

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SUMMARY

The gene *vestigial* has been proposed to act as a master gene because of its supposed capacity to initiate and drive wing development. We show that the ectopic expression of *vestigial* only induces ectopic outgrowths with wing cuticular differentiation and wing blade gene expression patterns in specific developmental and genetic contexts. In the process of transformation, *wingless* seems to be an essential but insufficient co-factor of *vestigial*. *vestigial* ectopic expression alone or *vestigial* plus *wingless* co-expression in clones differentiate 'mixed' cuticular patterns (they contain wing blade trichomes and chaetae characteristic of the endogenous surrounding tissue) and express wing blade genes only in patches of cells within the clones. In addition, we have found that these clones, in the wing imaginal disc, may cause autonomous as well as non-autonomous cuticular transformations and wing blade gene expression patterns. These non-autonomous effects in surrounding cells result from recruitment or 'inductive assimilation' of *vestigial* or *wingless-vestigial* overexpressing cells.

Key words: *Drosophila*, *vestigial*, Wing development, Inductive assimilation, Cellular identity, 'Mixed' tissues.

INTRODUCTION

Morphogenesis is normally associated with cell proliferation and genetic specification of territories or tissues. Several types of regulatory genes define these genetic specifications. Thus, in *Drosophila*, 'selector' genes such as *Ultrabithorax* (*Ubx*) or *engrailed* (*en*) (Garcia-Bellido, 1975), in combination with other selector genes, confer identity to segments or compartments. These genes are expressed in clonally restricted territories, are cell autonomous in genetic mosaics and transform the tissue to an archetypal specification. Other types of genes, such as *pannier* (*pnr*) (Calleja et al., 2000; Calleja et al., 1996), *iroquois* (*iro*) (Diez del Corral et al., 1999; Gomez-Skarmeta et al., 1996; Grillenzoni et al., 1998) or *Distal-less* (*Dll*) (Abu-Shaar and Mann, 1998; Campbell and Tomlinson, 1998; Gonzalez-Crespo et al., 1998; Wu and Cohen, 1999), may give territorial identity by themselves or in combination with others, but they specify territories that are rarely clonally delimited. 'Differentiation genes', such as *achaete-scute* (*ac-sc*), are involved in the terminal development of cells or tissues (Jimenez and Campos-Ortega, 1990). Finally, a new category has been introduced, the 'master' genes (Halder et al., 1995; Kim et al., 1996), which includes genes such as *eyeless* (*ey*) and *vestigial* (*vg*). These genes are considered to be able to individually initiate and drive specific developmental pathways, thus changing the fate of the tissues in which they are ectopically expressed. The concept of master gene has been applied to 'selector of tissue' genes later by other authors (Bray, 1999; Affolter and Mann, 2001; Halder and Carroll, 2001). In this context, the expression of *ey* or *vg* would be sufficient to give identity to eyes and wings, respectively (Halder et al., 1995; Kim et al., 1996). We show here that the ectopic expression of *vg* is the subject of temporal and genetic constraints in the promotion and driving of the wing developmental program.

vg encodes a nuclear protein of 453 amino acids with poor homologies to other known proteins. It is expressed at low levels in the primordial wing and haltere imaginal discs (Williams et al., 1991). Later in the development of both discs, the dorsoventral border of compartment is defined by differential expression of *apterous* (*ap*) (Diaz-Benjumea and Cohen, 1993), and subsequent restricted activation of *Notch* (*N*) (Irvine and Vogt, 1997) and downstream genes such as *wingless* (*wg*) (Kim et al., 1995). The activity of *wg* and *N* in the wing margin leads to the expression of the *vg* through the activation of the *vg* boundary enhancer (*vg BE-lacZ*) (Kim et al., 1996; Williams et al., 1994). Subsequent to *vg BE-lacZ* activation, the expression of *vg* in more proximal parts of the wing blade is regulated by the *decapentaplegic* (*dpp*) pathway and by *vg* itself, acting on the *vg* quadrant enhancer (*vg QE-lacZ*) (Kim et al., 1996). Reflecting the activity of the *vg* enhancers, Vg is expressed in a gradient with maximal concentrations in the wing margin and minimal in more proximal territories of the wing (Williams et al., 1991). Vg interacts with the product of the gene *scalloped* (*sd*), a protein with DNA recognition motifs (Campbell et al., 1992), forming a transcriptional activation complex (Halder and Carroll, 2001;

Halder et al., 1998). The Vg-Sd complex is known to regulate the expression of downstream genes involved in wing development (Halder and Carroll, 2001; Halder et al., 1998; Kim et al., 1996; Klein and Martínez-Arias, 1998). The absence of Vg, Sd or both causes lack of cell proliferation in the wing blade region where *vg* is expressed (Williams et al., 1991; Williams et al., 1993). By contrast, the ectopic expression of *vg* may cause the appearance of territories with cuticular and genetic expression patterns that are characteristic of distal wing blade (Halder et al., 1998; Kim et al., 1996). It is important to note that the ectopic expression of Sd alone does not induce tissue transformations. Thus, Sd is necessary to bind the complex Vg-Sd to DNA, but does not confer tissue specificity by itself (Halder and Carroll, 2001).

In order to analyse the capacity and the genetic requirements of the ectopic expression of *vg* to initiate and drive the transformation of tissue towards wing blade identity, we studied the autonomous and non autonomous cuticular and gene expression patterns that appear after the ectopic expression of *vg* during development. We drove *vg* ectopic expression using different territorial Gal4 lines (G4/UAS system) (Brand and Perrimon, 1993) or by Flip-out (FLP/FRT system) recombination in clones (de Celis and Bray, 1997; Ito et al., 1997). The results show that the morphogenetic effects of *vg* ectopic expression depend on developmental timing and the genetic specification of a disc territory where the overexpression takes place.

MATERIALS AND METHODS

Fly stocks

The experiments were carried out using the following G4 lines: dpp-G4 A.3 (Staehling-Hampton et al., 1994); vg-G4 (Simmonds et al., 1995); Dll-G4 (Calleja et al., 1996); c253-G4 (provided by Juan Modolell); pnr-G4 (Calleja et al., 1996); and patch-G4 (ptch-G4) (Speicher et al., 1994).

We used the following UAS lines: UAS-vg^{K} (Kim et al., 1996); UAS-vg^{Z} (Paumard-Rigal et al., 1998); UAS-*wg* (Lawrence et al., 1995); UAS-*wg* dominant negative (UAS-wg^{DN}) (Klein and Martínez-Arias, 1999); UAS-*Dll* (Gorfinkiel et al., 1997); UAS-homothorax-GFP (UAS-*hth*-GFP) (provided by Fernando Casares); UAS-*nubbin* (UAS-*nub*) (Neumann and Cohen, 1998); UAS-*sd* (Campbell et al., 1992); UAS-*Delta* (UAS-*Dl*) (Huppert et al., 1997); UAS-*Serrate* (UAS-*Ser*) and UAS-*Notch* constitutively active (UAS-$N^{intra12.1}$) (de Celis and Bray, 1997); UAS-*thickvein* constitutively active (UAS-tkv^{Q25}) (Lecuit et al., 1996) or dominant negative *tkv* (UAS-tkv^{DN}) (Haerry et al., 1998); UAS-*Ras* constitutively active (UAS-Ras^{V12}) (Karim and Rubin, 1998); UAS-*Ras* dominant negative (UAS-$Raf^{3.1DN}$) (Kim et al., 1995); UAS-P35 (provided by C. Lehner); UAS-*ap* (Fernandez-Funez et al., 1998); UAS-*fringe* (UAS-*fng*) (Kim et al., 1995); and UAS-GFP.

We used the following *lacZ* lines: *vg QE-lacZ* (Kim et al., 1996); *vg BE-lacZ* (Williams et al., 1994); *wg-lacZ* (Kassis et al., 1992); ap^{rk560} (*ap-lacZ*) (Diaz-Benjumea and Cohen, 1993); and sd^{ETX4} (*sd-lacZ*) (Anand et al., 1990).

The lines used for the induction of overexpression mosaics by Flip-out were *y* f^{36a} FLP^{122}*; abx/Ubx FRT* f^{+} *FRT G4 UAS lacZ* (de Celis and Bray, 1997); and *y* FLP^{122}*; Act FRT* y^{+} *FRT G4 UAS-GFP: MKRS/ SM6A-TM6B* (Ito et al., 1997).

Ectopic expression using territorial G4 lines and generation of overexpression clones

The ectopic expression with different lines G4 was induced at 17, 25 and 29°C. Genetic of Flip-out to induce clones of overexpression of *vg*, *wg* or both *wg-vg*: larvae were transferred from 25°C to 37°C for 7 minutes at different ages 36±12, 48±12 or 60±12 hours after laying egg AEL for *vg* clones, and 36±12 and 60±12 hours AEL for the *wg-vg* or *wg* clones.

Inmunohistochemistry

Imaginal disc were dissected in 1×PBS and fixed in 4% PFA at 4°C for 40 minutes, followed by 3×20 minute washes in 0.3% PBTriton and 3×20 minute washes in PBT-BSA. Incubation with primary antibody was carried out overnight at 4°C. After repeating washes with PBT and PBT-BSA the imaginal discs were incubated 2 hours at room temperature with the secondary antibody. Imaginal discs were mounted in Vectashield.

We used the following primary antibodies: rabbit anti-Dll and anti-Vg (provided by Sean Carroll); mouse anti-Wg (Hybridoma Bank); guinea pig anti-Hth (gift of Fernando Casares); mouse anti-Cut (Hybridoma Bank); mouse anti-Bs (provided by M. Affolter); mouse anti-Nub (provided by S. Cohen); mouse anti-En (Hybridoma Bank); rat anti-Ser (Hybridoma Bank); rat anti-CD2 (Hybridoma Bank); and mouse or rabbit anti β-gal (Amersham). We used rabbit and mouse Alexa 488, 546, Cy5 and guinea pig-Cy5 as secondary antibodies.

Microscopy and image treatment

For the processing of images in clear field and confocal microscopy we used the programs Metaview (Meta Imaging Corporation Plus) and Photoshop 6.0 (Adobe Corporation).

RESULTS

The ectopic expression of *vg* in G4 territories shows constraints on the promotion of the wing developmental program

We drove the ectopic expression of *vg* with different G4 lines, in order to observe the response of territories of cells ectopically expressing *vg* from early development onwards. Here, we monitor patterns of gene expression and cuticular differentiation.

It has been shown that the overexpression of *vg* driven by different G4 lines, such as ptch-G4 (Paumard-Rigal et al., 1998) (Simmonds et al., 1998), dpp-G4 (Kim et al., 1996; Klein and Martínez-Arias, 1998; Klein and Martínez-Arias, 1999) and Dll-G4 (Fig. 1A,C) (Halder et al., 1998; Weatherbee et al., 1998), ectopically induce histotypes and gene expression patterns characteristic of the wing blade. We explored the maximal transformation phenotypes using the driver Dll-G4. The ectopic expression of *vg* in the distal territories of the appendages (proboscis, first and second pairs of legs and genitalia), where *Dll* is expressed, leads to the transformation into tissues with characteristic cuticular patterns and differentiation of the wing blade (Fig. 1A,C). In the first and second pairs of legs, these transformations include typical trichomes, anteroposterior and dorsoventral wing margin chaetae (Fig. 1A2-A4, C1-C3), veins and campaniform sensillae (25/77) (Fig. 1A1,A3). In the third pair of legs, the transformations are to haltere histotypes (Fig. 1A5) (Halder et al., 1998; Weatherbee et al., 1998).

The adult transformations are correlated in imaginal discs with autonomous expression of genes characteristic of wing blade. These gene expressions only appear within GFP-expressing cells, as mobilised by Dll-G4. The ectopic wing margin differentiated in the outgrowths correlates with the

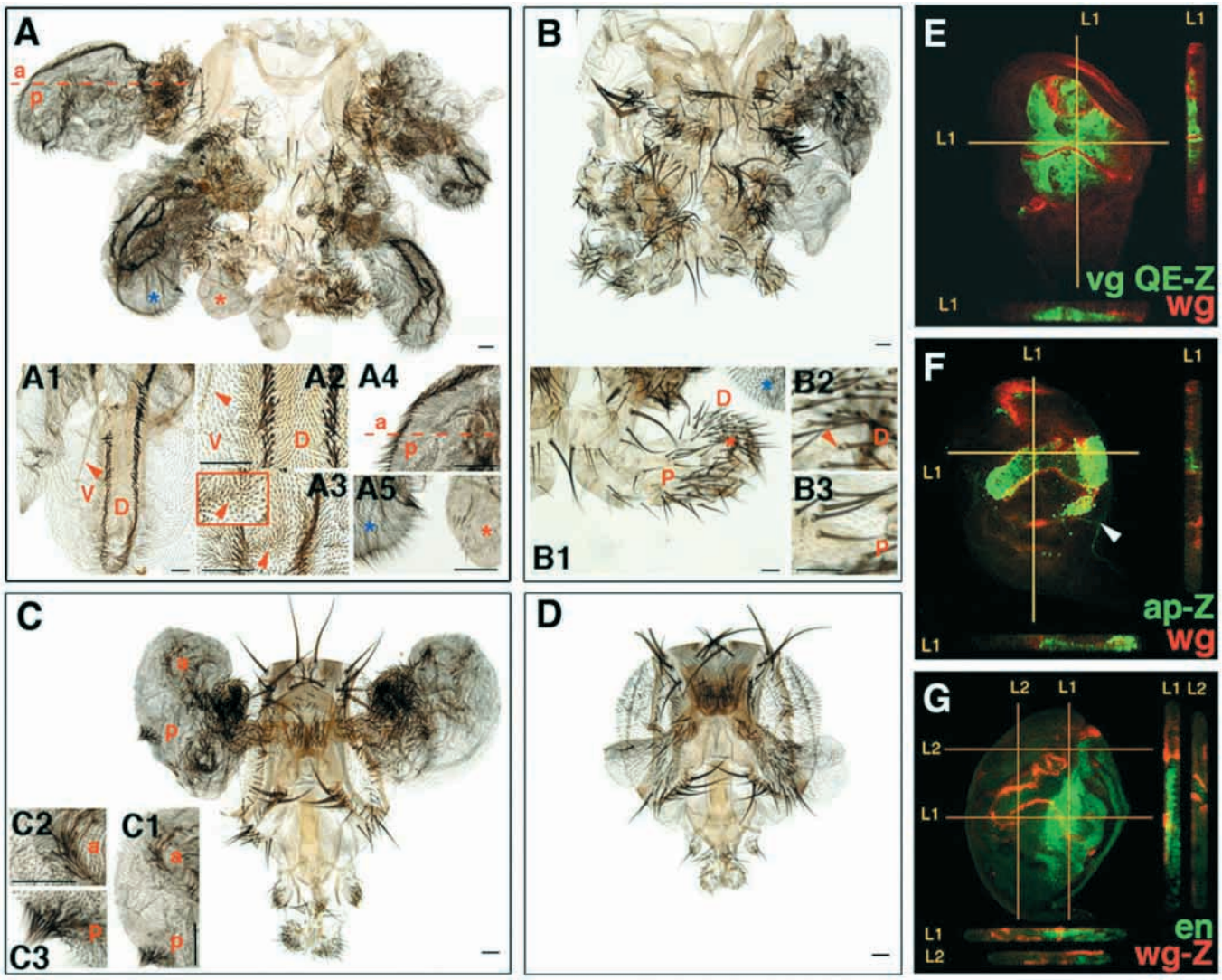

Fig. 1. Cuticular transformations caused by the ectopic expression of *vg* (A,C) and co-overexpression of wg^{DN}-*vg* (B,D) under the control of Dll-G4. Effects of *vg* ectopic overexpression on gene expression in the second pair of the legs driven by *Dll*-G4 (E-F). Lines indicate optical sections along the *z*-axis. Scale bars: 0.1 mm. (A) Ventral view of a transformed thorax with distal segments of first and second legs differentiating as wing blade (blue asterisk) and third legs as haltere (red asterisk). The broken red line separates anterior (a) from posterior (p) territories in transformed legs. (A1,A2) Transformed wing blade with dorsal (D) and ventral (V) regions. Arrowheads indicate veins. (A3) The arrowhead indicates a campaniform sensillae. (A4) Higher magnification of the wing margin in anteroposterior transition. (A5) Observe the histotypic differences between wing blade (blue asterisk) and haltere territories (red asterisk). (B) Ectopic co-expression of *vg* and a dominant-negative form of *wg* reduces the transformation phenotypes obtained with the ectopic expression of *vg* alone. (B1) Distal (D) (red arrowhead) and proximal (P) tarsal parts in the third pair of legs (red asterisk) at higher magnification. Distal segments (arrowhead indicates bracts) are shown in B2 and proximal segments (without bracts) in B3. (C) Ectopic expression of *vg* leads to transformation of antenna with anterior (a) and posterior (p) wing margin elements. The insets show the differentiation of wing margin elements with anterior (a; C1,C2) and posterior (p; C1,C2) specifications. (D) Transformation in the antenna is reduced by the co-expression of *vg* and a dominant-negative form of *wg*. (E) Ectopic expression of *vg QE-lacZ* (green) in the transformed legs. Notice repression of *vg QE-lacZ* report (green) in territories with high levels of *wg* expression (red). (F) Dorsal transformed territories in adult legs are correlated with *ap* (green) expression. Notice that the annular expression of ap-*lacZ* in the leg is modified: it is expanded in ventral territories but reduced (white arrowhead) in dorsal ones. The expression of *wg* (red) is activated at high levels in the border (wing margin) of *ap* expression. (G) Anterior and posterior specification in transformations denoted by the wild-type expression of *en* (green). The expression of *wg-lacZ* (red) corresponding to the wing margin appears in anterior as well as in posterior territories.

expression of *cut* (*ct*) (not shown) and *wg* in the presumptive wing margin in the disc (Fig. 1E-G). Thus, as in the wild-type wing margin, *wg* represses the expression of *vg QE-lacZ* (Fig. 1E). The transformations have large ventral wing territories and small dorsal territories encircled by the new wing margin (Fig. 1A1-4,E,F). The dorsoventral and anteroposterior transformations are correlated with the differential expression of *en* and *ap* (Fig. 1A,E,F,G), in the mature imaginal discs. The expression of *en* is maintained in the wild-type topology but the expression of *ap* is modified (Fig. 1F,G). Thus, the ring of

ap in the leg is repressed in dorsal leg territories and expanded in ventral ones (Fig. 1F). In the first and second leg-transformed territories, the genes characteristic of wing blade territories, such as *spalt* (*sal*), *blistered* (*bs*) (Halder et al., 1998; Weatherbee et al., 1998) and *vg QE-lacZ* (Fig. 1G) are expressed. In the third leg, where transformations are to haltere, some markers of wing transformation (such as *sal* or *vg QE-lacZ*) are not expressed (not shown), probably because of *Ubx* activity (Halder et al., 1998; Shashidhara et al., 1999; Weatherbee et al., 1998). *Ubx* expression is never modified by overexpression of *vg* or *wg-vg* driven by different G4 lines or in clones (not shown, see below). All described transformations and expression of wing blade genes are autonomously restricted to the *Dll* expression domain, visualised by GFP.

In contrast to these G4 lines, which induce transformation phenotypes, the ectopic expression of *vg* driven by G4 lines is not associated with histotypic transformations, and only causes tissue-specific malformations. Thus, with pnr-G4, we observe defects in thorax closure, and with c253-G4 duplications of chaetae in the notum (not shown). We also failed to obtain transformations when the ectopic expression of *vg* alone in the eye was driven by vg-G4.

These results indicate that expression of *vg* is necessary but not sufficient for the initiation of the wing blade developmental pathway.

Cooperative effects of *vg* and *wg* in the ectopic transformations

We analyzed why some G4 lines may lead transformations while others fail to do it driving UAS-*vg*. We have found that the overexpression of *vg* only induces transformations when the G4 line shares expression domains with high levels of *wg* in early stages of larval development. Thus, we have found that the overexpression of *vg* only induces transformations when the G4 line shares expression domains with high levels of *wg* in early stages of larval development. Furthermore, that *wg* is necessary for the augment the transformation is confirmed by experiments in the leg in which a dominant-negative form of *wg* (wg^{DN}) is ectopically co-expressed with *vg*, using the driver Dll-G4. In these legs, the transformation is strongly reduced (Fig. 1B,D). Without transformation, these legs maintain distal tarsal structures (territories of the legs with chaetae and without bracts), suggesting that the lack of transformation is not simply a consequence of the low levels of Wg activity (Fig. 1B). To test the hypothesis of collaboration of *wg* and *vg* in wing blade transformation, we co-expressed them in the expression domain of *vg*-G4 in the eye (Fig. 2B). Whereas the ectopic expression of *vg* or *wg* alone does not show cuticular transformations (not shown), the co-expression of both causes wing outgrowths with histotypical characteristics of wing blade (Fig. 2A). The transformation is autonomously associated with the expression of wing blade genetic markers as *nub*, *vg* (Fig. 2C,D) and *Dll* (not shown). Surprisingly, in the transformed territories the expression of *wg* is lower than we would expect of an overexpression using the G4/UAS system (Fig. 2D) (see Discussion). These results demonstrate that that *wg* and *vg* collaborate to initiate wing development in imaginal discs other than the wing.

Other experimental or genetic conditions that affect ectopic *vg* transformations

We have explored other experimental conditions that might allow *vg* to induce wing transformations.

We searched for other genes, in addition to *wg*, that might cooperate with *vg*, using vg-G4 expression in the eye. First, we studied: (1) the effects of one or several UAS-*vg* doses and G4 induction temperatures (not shown); (2) variations in the stoichiometry of Vg with Sd (Paumard-Rigal et al., 1998; Simmonds et al., 1998) co-expressing several doses of the corresponding UAS; (3) co-expression of *vg* with genes involved in the specification of the proximodistal axes of the wing, such as *nub*, *Dll* and *hth* (Abu-Shaar and Mann, 1998; Azpiazu and Morata, 2000; Cifuentes and Garcia-Bellido,

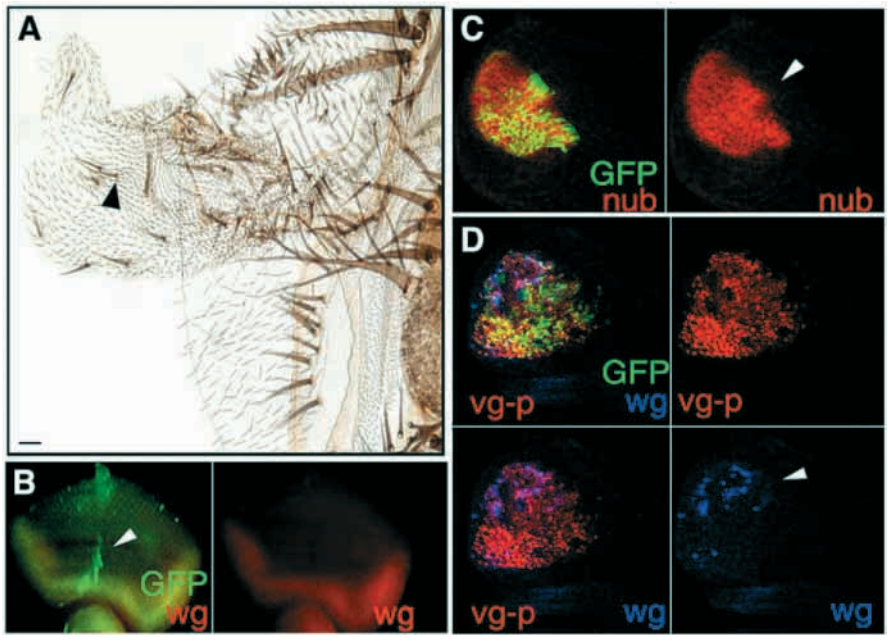

Fig. 2. Ectopic *wg-vg* co-expression driven by *vg-G4* causes the transformation of eye territories into wing blade with cuticular structures (A) and gene expression (C,D) characteristic of wing blade. The expression of *vg*-G4 is detected in the eye imaginal disc by the simultaneous mobilisation of UAS-GFP (GFP) (B-D). Scale bars: 0,1 mm. (A) Observe chaetae (black arrowhead) and wing blade trichomes which appear when we ectopically express *vg* in the eye. (B) In the wild-type eye imaginal disc, vg-G4 (GFP) and *wg* (red) are expressed in different territories. High levels of *wg* expression are detected in the poles of the eye, whereas vg-G4 is expressed in the equatorial line in the eye. (C) Notice the autonomous ectopic expression of *nub* (red; white arrowhead) in transformed eye cells caused by *wg-vg* co-expression (GFP). (D) Ectopic expression of *vg* (red; white arrowhead) in eye transformations caused by the *wg-vg* co-expression. Notice that the level of *wg* expression is very low in the transformed territories (white arrowhead).

1997); (4) co-expression with genes involved in dorsoventral wing margin specification, such as *ap* (Diaz-Benjumea and Cohen, 1993) and *fng* (Irvine and Wieschaus, 1994); (5) co-expression of *vg* with UAS p35 to rescue possible cell death (Hay et al., 1994); and (6) co-expression of *vg* with several constructs of genes that are involved in signalling pathways during development, such as UAS-*Dl*, UAS-*Ser*, UAS-$N^{intra12.1}$, UAS-tkv^{Q25}, UAS-Ras^{V12}, UAS-tkv^{DN}, UAS-$Raf^{3.1DN}$.

Secondly, we tested whether the size of the territory of ectopic expression was critical in the process of transformation, overexpressing *vg* with other G4 lines such as pnr-G4 and c253-G4.

We failed, in all instances, to enhance the histotypic transformation caused by overexpressing *vg* alone, and therefore conclude that neither the extension of the territory ectopically expressing *vg* nor the amount of overexpression is significantly relevant to the extent of transformation to wing. Thus, only *wg* in collaboration with *vg* seems to be specifically relevant in the promotion of wing development.

Phenotypes induced by the ectopic expression of *vg* in clones: temporal and genetic limitations to the initiation of a wing developmental program

Gene expression driven by a given G4 line occurs simultaneously in all the cells of the territory at a given developmental stage, allowing collaborative effects between cells. By contrast, the ectopic expression in clones provides temporal and positional limits to the transformation. In clonal mosaics, individual mutant cells are confronted with wild-type cells, allowing the study of autonomous and non-autonomous effects in cell proliferation, cuticular patterning and gene expression. We have monitored the cuticular and genetic effects of *vg* ectopic expression in Flip-out clones [labelled with *forked* (*f*) or GFP], in the wing, haltere, leg and eye-antenna imaginal discs.

In the wing blade and wing hinge, *vg* clones induce tubular, perpendicular outgrowths to the wing surface (Fig. 3A). The outgrowths include *vg*-expressing cells and surrounding wild-type cells (Fig. 3A). *vg* clones are frequently located at the tip of the outgrowth but they can also grow along the lateral zones (Fig. 3A). In those clones that appear in central parts of the wing, all the cells of the outgrowth and the clone always show a differentiation corresponding to wing blade trichomes (Fig. 3A). Clones near the wing margin may differentiate marginal chaetae (not shown). The size of the clones and the non-autonomous part of the outgrowth depend on their distance to the wing margin (Liu et al., 2000).

vg clones in the presumptive wing blade do not modify the wild-type expression of genes expressed, such as *Dll*, *bs* and *nub* (not shown). However in the wing hinge, clones of *vg* overexpression autonomously repress proximal genes such as *hth* (Fig. 3C) (Azpiazu and Morata, 2000; Casares and Mann, 2000; Liu et al., 2000) and activate antagonist distal genes such as *Dll* (Azpiazu and Morata, 2000; Liu et al., 2000), *vg QE-lacZ* (Fig. 3D), *nub* (Liu et al., 2000) and *bs* (Fig. 3C; Table 1)

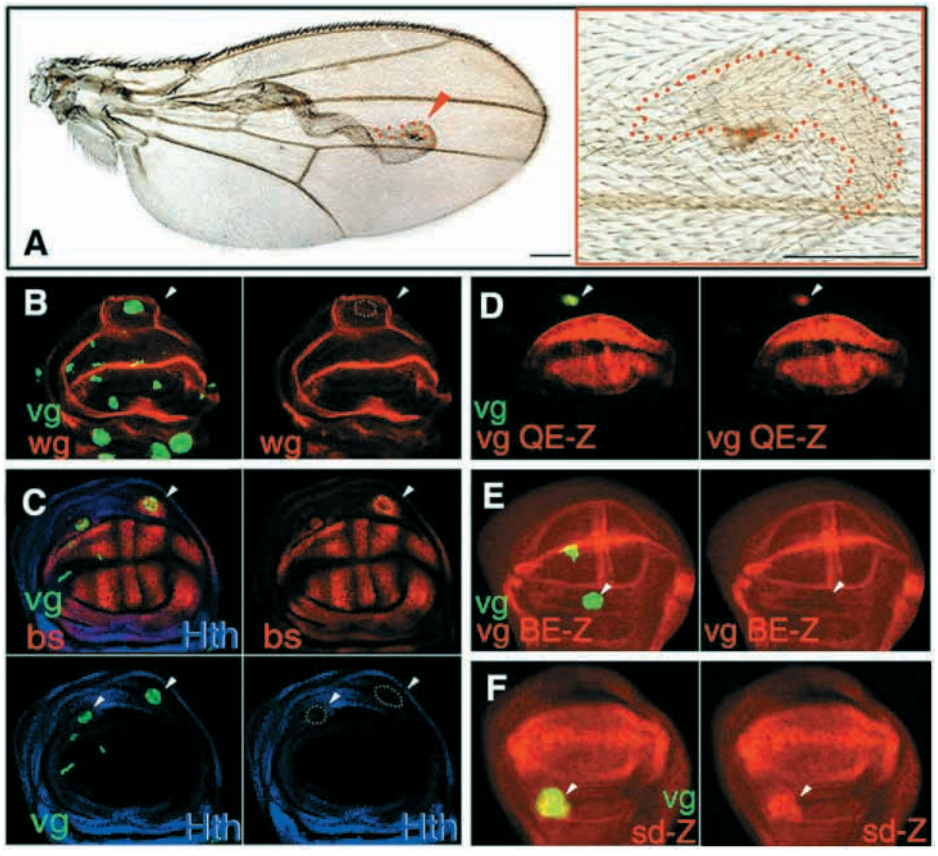

Fig. 3. Adult phenotypes (A) and imaginal disc gene expression (B-F) caused by the overexpression of *vg* in clones initiated in the wing blade and wing hinge. Adult clones are labelled with *f* and delimited by a broken red line. In the imaginal discs, *vg* clones are associated with green fluorescent protein (GFP). Scale bars: 0.1 mm. (A) Tubular outgrowth including a clone of *vg* (red arrowhead). The age of clone initiation is 36±12 hours AEL. In the inset a detail of the same clone is shown. (B) In the wing hinge, *vg* overexpression in clones (green) displaces the rings of *wg* expression (red) by several cellular diameters. The age of clone initiation is 60±12 hours AEL. (C) In the wing hinge, the *vg* overexpression in clones (green) non-autonomously activates the expression of Dll (red) and autonomously represses Hth expression (blue). The age of clone initiation is 60±12 hours AEL. (D) In the wing hinge, *vg* overexpression in clones (green) (white arrowhead) autonomously activates the *vg QE-lacZ* reporter (red). The age of clone initiation is 60±12 hours AEL. (E) *vg* overexpression in clones (green; white arrowhead) never activates the *vg BE-lacZ* reporter (red) reporter in the wing imaginal disc (or in other tissues). The age of clone initiation is 60±12 hours AEL. (F) In all studied tissues, *vg* overexpression in clones (green; white arrowhead) autonomously activates the *sd-lacZ* reporter (red). The age of clone initiation is 60±12 hours AEL.

(Liu et al., 2000). In some cases, the ectopic expression of distal genes may appear non-autonomously outside the clone, up to a distance of several cell diameters (Fig. 3B,C; Table 1) (Liu et al., 2000). Vg, or the *vg* enhancer *lacZ* reporters, are never detected non-autonomously in *vg* clones located outside of the wing blade. Whereas early *vg* clones can show co-expression with *wg* (not shown), later in development, *wg* expression is displaced outside of the clone several cell diameters (Fig. 3A) (Liu et al., 2000). The absence of wing margin cuticular elements in adult *vg* clones and *ct* or *vg BE-lacZ* (Fig. 3E; Table 1) expression in the discs suggests that the clones are specified as wing blade, not including wing margin territories. *vg* clones in the wing imaginal disc show a correlated and autonomous expression of *sd-lacZ* within the clone (Fig. 3F). In tissues other than the wing, the ectopic expression of *vg* may drive the expression of its transcriptional partner (not shown) (Halder and Carroll, 2001; Halder et al., 1998).

In territories other than the wing, *vg* clones show transformations toward wing histotype only when they are initiated in specific positions and stages of development. Thus, ectopic expression of *vg* in clones is only associated with wing outgrowth phenotypes when it is initiated in territories that normally express high levels of *wg* (Fig. 4C, Fig. 5C,H). For example, *vg* clones in the notum, only show wing histotype when they are initiated very early in development (36±12 hours AEL), whereas in the eye and leg imaginal discs, transformations may appear later (48±12 hours AEL). Clones initiated later in development or in territories with low levels of *wg* expression cause cuticular abnormalities (Fig. 5B) but not transformations towards wing histotype.

In tissues other than the wing, clones of *vg* ectopic expression associated with transformation differentiate only wing blade trichomes (Fig. 4A,B, Fig. 5A,G), in contrast to the overexpression of *vg* with G4 lines in the same territories. The wing blade trichomes in some tissues such as notum or legs may appear 'mixed', with tissue-specific chaetae in a 'salt and pepper' distribution (Fig. 4B, Fig. 5A). The adult 'mixed' cuticular patterns are correlated for each examined tissue with the specific expression of some wing blade genes (Table 1). Thus, we never detected the expression of *nub* in the notum, whereas it is induced in the leg or eye imaginal discs (Fig. 5I); and *bs* is never detected in the eye, whereas it is induced in the leg (Fig. 5D). Paradoxically, in contrast to 'salt and pepper' distribution of the adult cuticular structures detected in the transformations, the ectopic expression of specific wing blade genes only occurs in subsets of cells within the clones (Fig. 4D, Fig. 5D,E). Moreover, the cells expressing wing blade genes are compacted and located anywhere within the clones of *vg* (Fig. 4D, Fig. 5D,E). The discrepancy between cuticular pattern and gene expression suggests that *vg* can not displace all endogenous identity signals or, alternatively, that there are non-autonomous influences of surrounding cells on of the clone expressing wing blade genes.

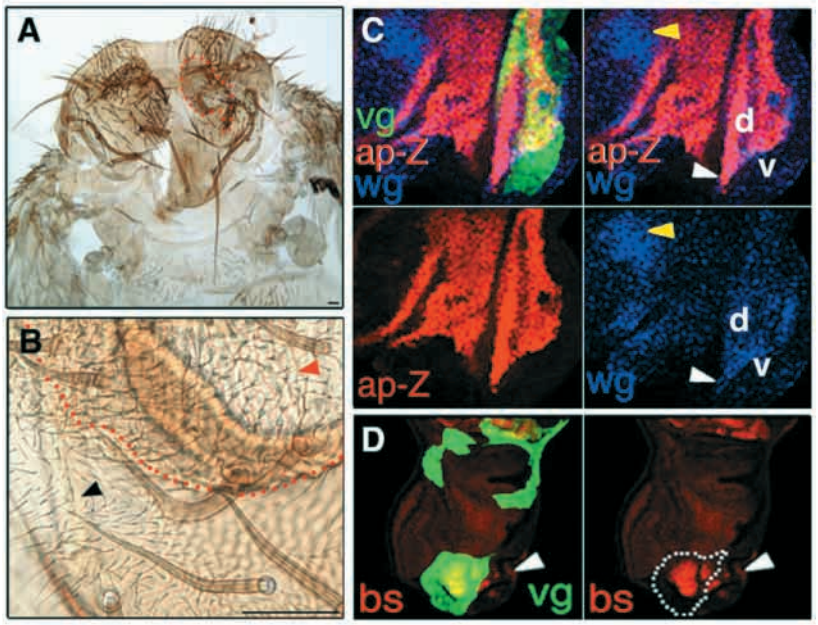

Fig. 4 . Adult 'mixed' tissue phenotypes (A,B) and imaginal disc expression (C,D) caused by the ectopic clonal expression of *vg* in the notum. Adult clones are labelled with *f* and delimited by a broken red line. In the imaginal discs, *vg* clones are associated with GFP. The age of clone initiation is 36±12 hours AEL. Scale bars: 0.1 mm. (A) Clone of *vg* ectopic expression in the notum initiated at 36±12 hours AEL; high magnification is shown in B. The clone territory contains trichomes of wing blade (red arrowhead) and notum chaetae in a 'salt and pepper' distribution. Notice the differences between trichomes with wing blade characteristics (red arrowhead) contained in the clone with notum trichomes (black arrowhead). (C) Clones of *vg* ectopic expression (green) in the notum that simultaneously contain cells expressing [dorsal territories (d)] and non-expressing [ventral territories (v)] the *ap-lacZ* reporter. Notice that endogenous *wg* expression in the notum appears displaced (yellow arrowhead), whereas *wg* expression is enhanced within the clone (white arrowhead) at the confrontation between cells expressing and non-expressing *ap-lacZ*, as occurs in the wild-type wing margin. (D) Adult mixed tissues are correlated, in imaginal discs, with the autonomous expression of wing blade genes such as *bs* (red). Notice that the expression of *bs* is non-autonomously induced outside the clone (green; white arrowhead), as well as partially induced within the clone.

In tissues other than the wing, the histotype transformations and expression of wing blade gene are cell autonomous. But in the notum, *vg* clones that straddle the DV boundary and are initiated in territories with high levels of *wg* (Fig. 4C) and occasionally cause non-autonomous expression of wing gene markers such as *bs* (Fig. 4D). This phenomenon of non-autonomous induction of tissue to express wing blade genes, we called 'inductive assimilation' (see Discussion). In these clones, the wild-type expression of *wg* is displaced, but where ap expressing and non-expressing cells are confronted in the clone, *wg* expression is autonomously enhanced, as in the wing margin (Fig. 4C). This reflects the possibility that *vg* clones may recruit the expression of *ap* before the wild-type specification of DV wing margin, generating ectopic DV wing margins.

As in the G4 experiments, the expression of *en* is not modified in *vg* clones, and the disc therefore retains the embryonic AP compartment specification. However, *ap* expression is altered in clones expressing ectopically *vg* in a tissue-

specific way. Thus, *ap* expression is not modified in the wing imaginal disc or haltere, whereas in leg discs, *ap* expression is induced in ventral territories and repressed in dorsal ones (Fig. 5E,F).

Similar to *en* expression, *Ubx* is not modified in clones expressing ectopically *vg* or *vg* and *wg* simultaneously (*wg-vg*), and therefore, the segmental identity dependent on *Ubx* expression is maintained. In the haltere, *vg* or *wg-vg* clones have otherwise the same autonomous and non-autonomous effects than in the wing (not shown).

The activity of *wg* pathway together with *vg* is insufficient to promote a wing developmental program in clones

In order to test the interaction of the *wg* pathway with *vg* in clones, we have studied the cuticular and gene expression patterns shown by cells expressing either *vg* or *wg* alone, and co-expressing *wg* and *vg* (*wg-vg*) simultaneously. The phenotypes of *wg* or *wg-vg* clones have been monitored in the wing, haltere, leg and eye-antenna imaginal discs.

In the wing, the overexpression of *wg* in clones does not cause outgrowths and all cells of the clone differentiate into wing margin sensory elements (not shown). These results confirm the proposition of Klein and co-workers (Klein et al., 1997; Klein and Martínez-Arias, 1998) that the overexpression of *wg* induces the cells to acquire characteristics of wing margin. However, these cells fail to express *vg BE-lacZ* or other wing margin genes such as *ct* (not shown). In these clones, some wing blade markers such as *Dll* (not shown) (Zecca et al., 1996), *vg* (Fig. 6B) (Zecca et al., 1996) or *nub* (Fig. 6C)

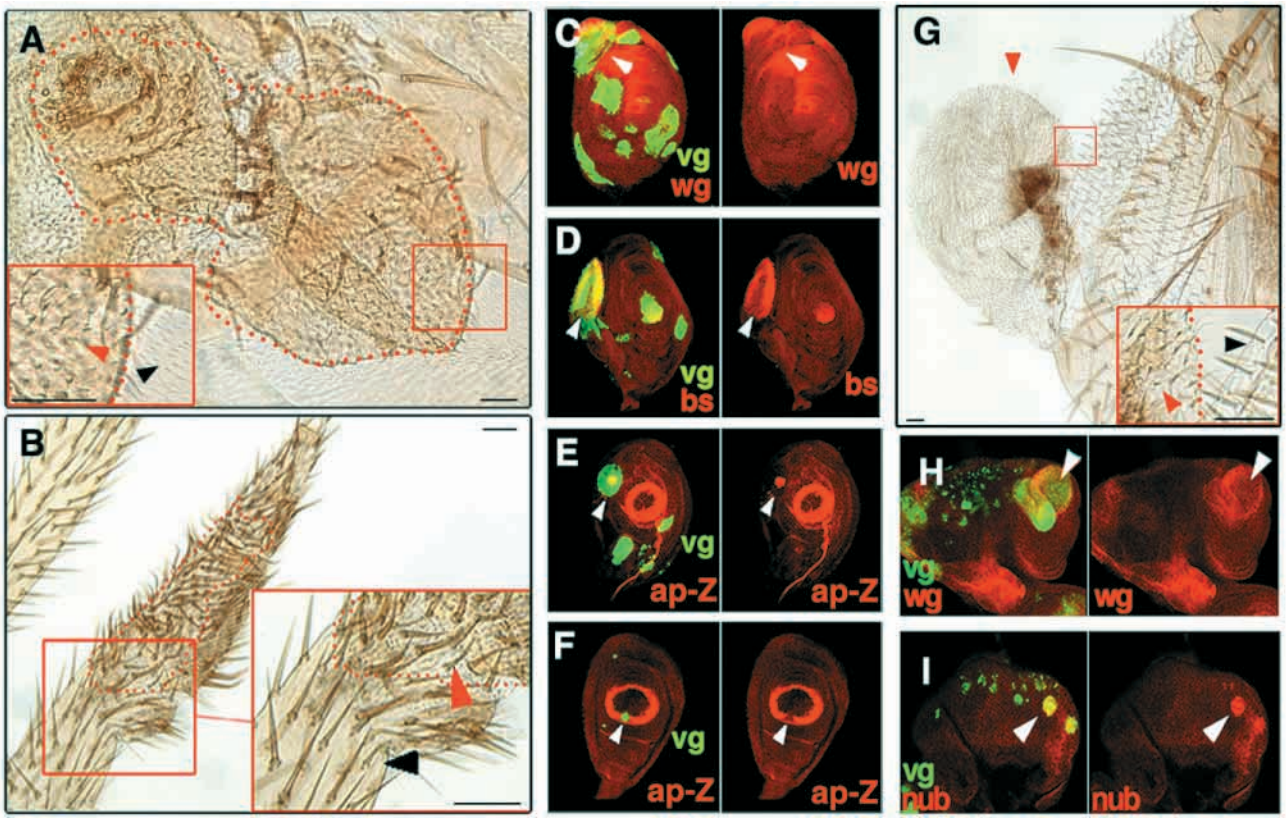

Fig. 5. Adult phenotypes (A,B,G) and gene expression patterns caused by the ectopic expression of *vg* in clones located in the leg (C-F) and eye (H,I) imaginal discs. Adult clones are labelled with *f* and delimited by a broken red line. In the imaginal discs, *vg* clones are associated with GFP. Scale bars: 0.1 mm. (A) Clones of *vg* ectopic expression include 'mixed' cuticular differentiation patterns in the leg, with leg chaetae mixed in a 'salt and pepper' distribution with wing blade trichomes. In the inset, compare trichomes of the leg (black arrowhead) with wing blade trichomes (red arrowhead). The age of clone initiation is 36±12 hours AEL. (B) Proximalisation phenotypes of the leg without cuticular transformation induced by *vg* ectopic expression in clones. In distal tarsal segments of the leg, typical chaetes are associated with bracts (black arrowhead in the inset), whereas in *vg* clones chaeates are not associated with bracts (red arrowhead in the inset). Notice the non-autonomous size reduction in distal tarsal segments of the leg. The age of clone initiation is 36±12 hours AEL. (C) Clones of *vg* ectopic expression (green) autonomously reduce endogenous *wg* expression (red; white arrowhead). The age of clone initiation is 60±12 hours AEL. (D) Clones of *vg* ectopic expression (green) may activate *bs* (red) expression (white arrowhead) in a subset of cells within the clone. Notice that only those clones situated in ventral leg territories induce *bs* expression. The age of clone initiation is 60±12 hours AEL. (E,F) Clones of *vg* ectopic expression (green) in ventral territories of the leg show autonomous expression of the *ap-lacZ* reporter (red) (E), whereas it is repressed in dorsal territories (F). Notice that *ap* expression is not detected in all cells of the clone, similar to D. The age of clone initiation is 60±12 hours AEL. (G) Outgrowth with wing blade trichomes (indicated by red arrowhead) induced by *vg* clone. In the inset, trichomes with wing blade characteristics (red arrowhead) are compared with surrounded ommatidial differentiation (black arrowhead). The age of clone initiation is 36±12 hours AEL. (H) Clones of ectopic *vg* expression (green) autonomously reduce the expression of *wg* (red; white arrowhead). The age of clone initiation is 60±12 hours AEL. (I) In the eye, clones of *vg* ectopic expression (green) autonomously induce the expression of *nub* (white arrowhead). Only those clones induced in territories with high levels of Wg express *nub*. The age of clone initiation is 60±12 hours AEL.

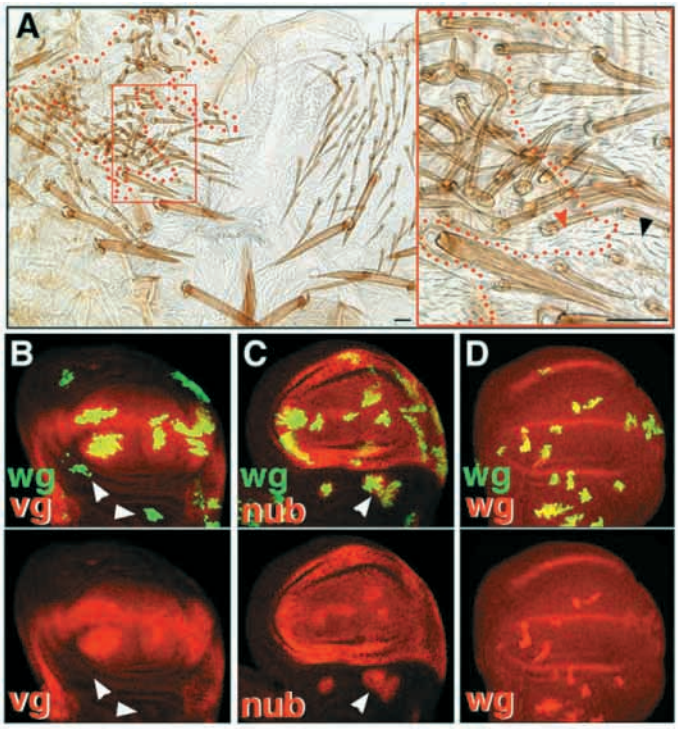

Fig. 6. Cuticular phenotypes (A) and imaginal disc expression pattern (B-D) caused by clonal overexpression of *wg* in the wing imaginal disc. Adult clones are labelled with *f* and delimited by a broken red line. In the imaginal discs, *wg* clones are associated with GFP. Scale bars: 0.1 mm. (A) Clones of *wg* overexpression in the notum show the typical differentiation of this territory. In the inset notice that trichomes are equal inside (red arrowhead) and outside the clone (black arrowhead). The age of clone initiation is 36±12 hours AEL. (B) Clones of *wg* overexpression (green) in the wing blade autonomously and non-autonomously induce the expression of *vg* (red), whereas those clones outside the wing blade do not express *vg* (white arrowhead). The age of clone initiation is 60±12 hours AEL. (C) Clones of *wg* overexpression (green) in the wing imaginal disc autonomous and non-autonomously induce the expression of *nub* (red; white arrowhead). Notice that non-autonomous expression of *nub* is detected only in the closest cells surrounding the clone. The age of clone initiation is 60±12 hours AEL. (D) Clones of *wg* overexpression (green) show homogenous and high levels of Wg (red). The age of clone initiation is 60±12 hours AEL.

are autonomous and non-autonomously expressed, whereas other wing blade genes such as *bs* are autonomous and non-autonomously repressed (not shown).

In the notum, overexpression of *wg* in clones is not associated with histotype transformations or 'mixed' tissues (Fig. 6A), but may autonomously and non-autonomously express wing blade genes such as *Dll* (not shown) and *nub* (Fig. 6C) (Table 1). In *wg* clones, the non-autonomous gene expression is restricted to the nearest surrounding cells of the clone (see below and Discussion). The absence of transformation detected in these clones overexpressing *wg* is correlated with the absence of *vg* expression (Fig. 6B).

In the eye or leg imaginal discs, overexpression of *wg* in clones usually does not activate the ectopic expression of *vg* or show cuticular transformations (Table 1), but causes specific cuticular perturbations and gene expression alterations as shown elsewhere (Lee and Treisman, 2001; Royet and Finkelstein, 1997; Struhl and Basler, 1993; Theisen et al., 1996).

In the wing blade, the co-expression *wg-vg* in clones leads to the formation of tubular and perpendicular outgrowths. The size of the outgrowths is larger than in *vg* clones and is dependent on the distance from the wing margin (Fig. 7A,B). All the cells of the *wg-vg* clones are differentiated into wing margin sensory elements, as occurs in *wg* clones, whereas non autonomous territories of the outgrowth are differentiated into wing blade trichomes (Fig. 7A,B). In contrast to clones of *wg*, in which we detect homogenously high levels of Wg (Fig. 6D), clones of *wg-vg* show low levels of Wg in some cases (Fig. 7E; Table 1), which are still sufficient to promote the autonomous differentiation of wing margin sensory elements. *wg-vg* clones do not express *vg BE-lacZ* or *ct* (not shown) (Table 1). In the wing blade, the activation or repression of wing blade genes is equal to that observed in the *wg* overexpression clones.

In contrast to clones of either *vg* or *wg* alone, *wg-vg* clones in the wing hinge and notum cause transformation phenotypes everywhere. All the cells autonomously differentiate into wing margin and non-autonomously differentiate into wing blade trichomes (Fig. 7C). These transformations are correlated with

Table 1. Effects of *vg*, *wg* or *wg-vg* clones on gene expression patterns in different territories

Genotype	Territory	Tested genes											Transformed tissues (Adult phenotype)
		nub	bs	vg QE	vg BE	sd-Z	Dll	Hth	wg	ct	ap	en	
UAS vg	Wing-hinge	▲	▲	▲	■	▲	▲	▼	▼	■	■	■	Wing blade without D/V margin
	Notum	■	▲	★	■	▲	▲	▼	▲▼	■	■	■	Mixed
	Leg	▲	▲	■	■	▲	▼	▲	▼	▼	▲▼	■	Mixed
	Eye	▲	■	▲	■	▲	▲	▼	▼	▼	■	■	Wing blade without D/V margin
UAS wg; UAS vg	Wing-hinge	▲	▲ ▼	▲ ■	■	★	▲	▼	▲▼	■	■	■	D/V margin
	Notum	▲	■	▲	■	★	▲	▼	▲▼	■	■	■	D/V margin
	Leg	▲▼	▲■	■	■	★	▼	▲	▲▼	■	▲▼	■	Mixed
	Eye	▲	■	▲	■	★	▲	▼	▲▼	■	■	■	D/V margin
UAS wg	Wing-hinge	▲	▼	■	▲■	★	▲	★	▲	■	★	★	Not transformed
	Notum	▲	■	■	■	★	▲	★	▲	■	★	★	Not transformed
	Leg	★	■	■	■	★	▲■	★	▲	■	★	★	Not transformed
	Eye	■	■	■	■	★	★	★	▲	■	★	★	Not transformed

▲ Autonomous activation; ▲ Non-autonomous activation; ▼ Autonomous repression; ▼ Non-autonomous repression; ■ wildtype expression; ★ Not studied

the autonomous and non-autonomous expression of the wing blade genes studied (Fig. 7D,F,G; Table 1). In *wg-vg* clones, the expression of *vg* and other wing blade genes is autonomous and non-autonomous, but, in *wg-vg* clones the non-autonomous expression is extended to larger cell distances surrounding the clone than in *wg* clones (compare Fig. 6C with Fig. 7F and Table 1). In contrast to *vg* clones, the expression of *bs* in *wg-vg* clones is reduced, possibly because of their genetic specification as similar to cells of the wing margin region (Fig. 7G; Table 1).

In imaginal discs other than the wing, the co-expression of *wg-vg* in clones shows 'mixed' phenotypes (Fig. 8A,B) and gene expression specificities similar to clones expressing *vg* ectopically (Fig. 8C,D; Table 1), again revealing regional restrictions to the induction of transformations and specific limitations of tissue to activation of wing blade gene expression. In contrast to clones of *wg* alone, *wg-vg* clones show transformation phenotypes, probably because of the presence of *vg* expression. As in *vg* overexpression clones, *wg-vg* clones modify neither *ap* nor *en* expression (Table 1). These results suggest that the co-expression of *wg-vg* remains insufficient to promote a wing developmental program outside the wing imaginal disc.

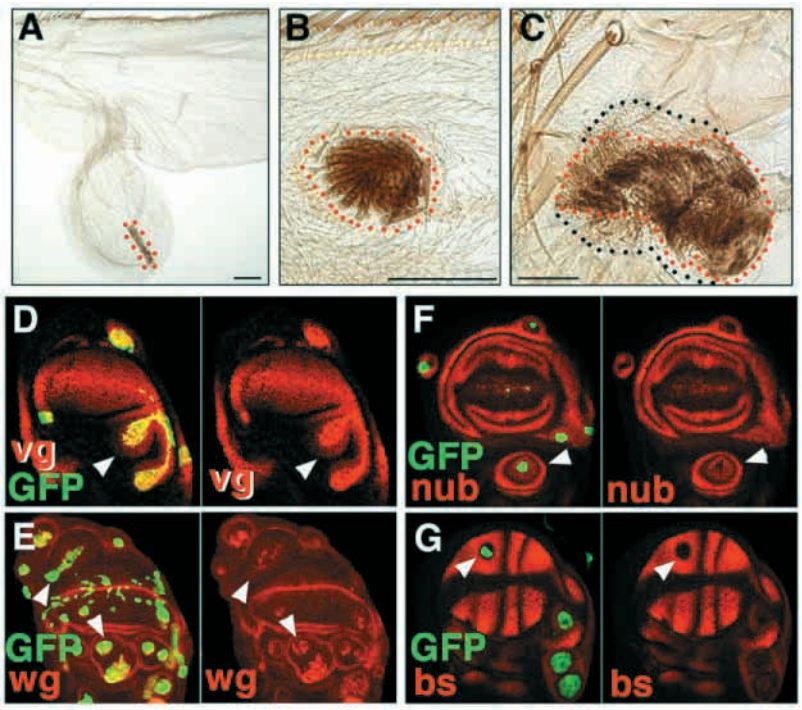

Fig. 7. Adult phenotypes (A-C) and imaginal disc expression patterns (D-G) caused by the co-expression of *wg* and *vg* in clones in the wing imaginal disc. Adult clones are labelled with *f* and delimited by a broken red line. In the imaginal discs, *wg-vg* clones are associated with GFP. Scale bars: 0.1 mm. (A) *wg-vg* clones in the wing blade may induce the appearance of tubular outgrowths. Notice the large non-autonomous growth induced by the clone. The age of clone initiation is 60±12 hours AEL. (B) Clones situated near the wing margin do not induce outgrowths. The age of clone initiation is 60±12 hours AEL. (C) In the notum, all the cells of *wg-vg* clones (delimited by a broken red line) differentiate into wing margin sensory elements and non-autonomously induce cuticular transformation to wing blade (delimited by a broken black line). The age of clone initiation is 36±12 hours AEL. (D) *wg-vg* clones (GFP) induced anywhere within the imaginal disc activate autonomously and non-autonomously the expression of *vg* (red; white arrowhead). The age of clone initiation is 60±12 hours AEL. (E) *wg-vg* (GFP) clones show heterogeneous or low levels of *wg* expression (red; white arrowheads). The age of clone initiation is 60±12 hours AEL. (F) *wg-vg* clones (GFP) autonomous and non-autonomously activate the expression of *nub* (red) anywhere in the imaginal disc. Notice that the non-autonomous expression of *nub* (red) is detected in cells surrounding the clone at long distances. The age of clone initiation is 60±12 hours AEL. (G) *wg-vg* clones (GFP) in the wing blade autonomously and non-autonomously repress *bs* (red; white arrowhead) expression, but in the wing hinge or in the notum, *bs* is autonomously repressed and non-autonomously induced. The age of clone initiation is 60±12 hours AEL.

DISCUSSION

The notion of 'master gene', as applied to the gene *ey* (Halder et al., 1995), corresponds to a gene that by itself would trigger a developmental program that is independent of the tissue where it is expressed. Although this definition has been applied to *vg* (Kim et al., 1996), the present results indicate otherwise. The ectopic expression of *vg* elicits certain characteristics of 'wing blade' development but is not sufficient for a complete transformation. The effect of *vg* depends on the time and genetic context of the tissue where it is overexpressed. Our results, according to other authors (Klein and Martínez-Arias, 1999), reveal a strong dependence of *vg* on *wg* to initiate a wing blade developmental pathway. Wg by itself does not lead to tissue transformations. This cooperative effect between *wg* and *vg* remains insufficient in all tissues analysed, suggesting the existence of additional genes necessary to initiate and drive wing development. We do not know the molecular mechanisms that underlie the interaction between *wg* pathway and *vg*. However, the co-expression of *vg* with a construct of *armadillo* (*arm*) (transcriptional effector of *wg* pathway) using vg-G4 fails to promote the transformation of eye tissue (preliminary data, not shown). This result suggests that the interaction of *wg* and *vg* takes place upstream of *arm* and, therefore, outside of the cell nucleus. Whereas *vg* requires high levels of *wg* expression to initiate wing development, the clones of *vg* overexpression contain in later stages, low or null levels of *wg* expression. Moreover, *wg-vg* co-overexpression clones can also show low levels of *wg*, even when *wg* is also mobilised in G4 territories or in Flip-out clones. These results suggest that *vg* may indirectly reduce *wg* expression once wing development is already initiated, and may explain why the transformed tissue in *vg* clones does not contain wing margin cuticular elements. The late repression of *wg* seems to be important to specify territories of the wing blade depending on *vg* expression outside of the wing margin; if high levels of Wg are maintained all cells differentiate into wing margin chaetae. We conclude that *wg* and *vg* activities together specify wing margin territories, but *vg* alone specifies the remaining part of the wing blade.

The ectopic expression of *vg* or *wg-vg* in clones may cause

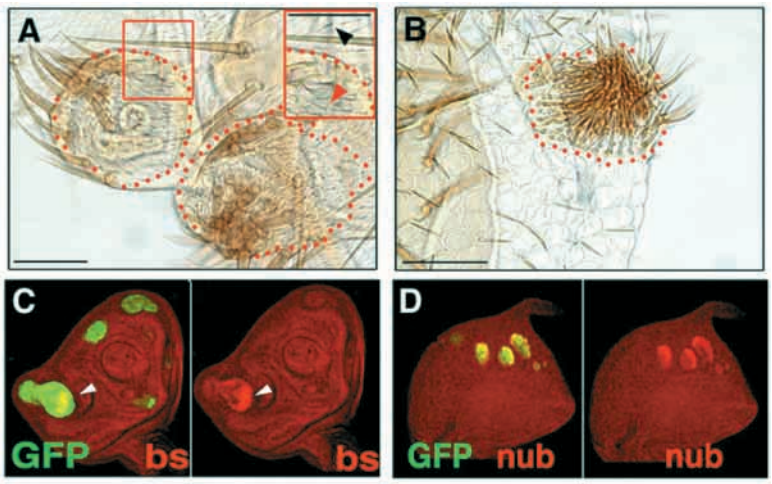

Fig. 8. Adult phenotypes (A,B) and imaginal disc expression patterns (C,D) caused by the ectopic clonal co-expression of *wg* and *vg* in the leg (A,C) and eye (B,E) imaginal discs. Adult clones are labelled with *f* and delimited by a broken red line. In the imaginal discs, clones are associated with GFP. Age of clone initiation is 60±12 hours AEL. Scale bars: 0.1 mm. (A) *wg-vg* clones induce a 'mixed' cuticular differentiation pattern in the leg. Notice that in the outgrowth chaetae characteristic of the leg appear mixed with wing blade trichomes in a 'salt and pepper' distribution. The inset shows the differences between wing blade trichomes of the clone (red arrowhead) and leg trichomes outside the clone (black arrowhead). (B) In the eye, all cells of the *wg-vg* clones autonomously differentiate into sensory elements of the wing margin. (C) In the leg, *wg-vg* clones activate the expression of wing blade genes such as *bs* (red), only in a subset of cells within the clones (arrowhead). Notice that the expression of *bs* is not detected in all the clones. (D) In the eye, *wg-vg* clones (GFP) autonomously activate the expression of *nub* (red) in all positions.

outgrowths with wing histotypic characteristics or patterning perturbations in the notum, leg or eyes. The transformed tissues show 'mixed' phenotypes or 'mosaic' territories where, in a 'salt and pepper' distribution, wing blade trichomes co-exist with notum or leg chaetae. Adult cuticular 'mixed' phenotypes are correlated with the ectopic expression of wing blade genes in particular combinations (Table 1). However, expression of wing blade genes is detected only in some compact groups of cells within the clones. These results indicate that either *vg* or *wg-vg* are insufficient by themselves to displace all endogenous signals of identity, or that reciprocal non-autonomous influences between clonal cells and surrounding cells exist, reducing the expression of wing blade genes to groups of cells within clones. The change of wing blade genes expression in compact groups of cells in the disc and 'mixed' (salt and pepper) cuticular phenotypes in the adult could result from cell interactions during patterning and cell rearrangements in pupal stages.

Transformations induced by overexpression of *vg* or *wg-vg* in clones and G4 territories are, as a rule, cell autonomous, except in the wing hinge, notum and corresponding tissues in the haltere. In the wing hinge the cells of the outgrowths outside the *vg* clones differentiate into wing blade territories and show gene expression patterns characteristic of the wing blade cells located between the proximal *vg* expression and the internal ring of *wg* in the wild-type disc. This suggests that the non-autonomous effects in *vg* clones could reproduce the wild-type intercalary growth induced by the confrontation of cells expressing proximal genes with distal genes. In the notum, *vg* clones located simultaneously in territories expressing and not expressing *ap*, and initiated in the *wg* expression domain, may non-autonomously recruit surrounding cells to express characteristic wing blade genes at long cell distances, as *wg-vg* clones do. Thus, *vg* together with *wg* expression is necessary to induce and extend the transformation over long distances outside the clones. In contrast to *vg* or *wg-vg* clones, *wg* clones do not show non-autonomous transformation phenotypes and expression of wing blade genes at long distances. The issue of whether the recruitment process is caused by Wg diffusion, or whether it results from intercalary growth induced by the confrontation between cells expressing proximal genes (genes of the notum) and cells expressing distal genes (wing blade genes), remains unresolved.

The expression of selector genes like *Ubx* and *en* is not modified by overexpression of *vg* or *wg-vg*, but is inherited and maintained. However, the expression of the selector gene *ap* can be modified or inherited in some tissues, such as the legs, to give DV identity.

The comparative analysis of *vg* with other morphogenetic genes suggests that *vg* acts as *Dll*, *pnr* or *iro*, rather than as a 'master' or 'selector of tissue' gene: *vg* is simply a component of the genetic combination that is necessary to initiate and drive wing blade development where *vg* is normally expressed. Interestingly, the function of *vg*, in addition to conferring territorial identity, may also non-autonomously recruit surrounding cells ('inductive assimilation'), changing their specific cuticular and gene expression patterns. This is related to its function as a local organiser of growth when it is expressed among cells with different positional or regional fates. Later in development, *vg*, in combination with other genes, activates an inventory of downstream wing genes that specify more discrete territories within the wing blade such as veins, interveins and sensory elements.

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