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The Vestigial gene product provides a molecular context for the interpretation of signals during the development of the wing in *Drosophila*

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SUMMARY

The *vestigial* (*vg*) gene of *Drosophila* plays a central role in the development and patterning of the wing: loss of *vestigial* results in failures in wing development and ectopic expression of *vestigial* leads to the development of ectopic wings. The wing-specific regulation of vestigial is mediated through two enhancers: (1) the Boundary Enhancer (*vg*BE) is early acting and becomes restricted to the wing margin, and (2) the Quadrant Enhancer (*vg*QE), acts later and is responsible for the expression of vestigial in the developing wing blade. These enhancers receive regulatory inputs from three signalling pathways: *wingless*, *decapentaplegic* and *Notch*/*Suppressor of Hairless*. Our experiments show that the vestigial gene product is also an input in the regulation of vestigial expression. In particular, Vestigial provides an important input for the regulation of the activity of the *vg*QE acting in concert with Wingless and Decapentaplegic. Our results suggest how interactions between *vg*BE and the *vg*QE mediated by Vestigial can explain the interactions between the wing margin and the wing blade during the growth of the wing. We further show that Vestigial and Notch collaborate with Wingless to subdivide and pattern the wing blade. These results lead us to propose a general role for Wingless during development in which it stabilizes cell fate decisions that have been implemented by other molecules.

Key words: *Drosophila*, Vestigial, Wing, Boundary Enhancer, Quadrant Enhancer, Notch, Wingless, Decapentaplegic

INTRODUCTION

The development and patterning of structures such as teeth, limbs, heart or liver are complex processes that rely on the spatial and temporal orchestration of cell interactions. These in turn rely on signalling systems but, in the last few years, it has become clear that a specific structure is not the outcome of specific signalling events. On the contrary, the available information suggests that there is a limited number of signalling systems which often work through a small number of effectors to elicit a great variety of responses. The specificity of the outcome resides on the effectors available to the signalling system through the history of the cell. For example, in vertebrates, Sonic Hedgehog (Shh) is involved in the development and patterning of the limb, the specification of the floor plate, the determination of neuronal cell fates and other processes (Riddle et al., 1993; Echelard et al., 1993). For each of these functions, Shh controls specific sets of genes. However, it always does it through the same signal transduction pathway and therefore something has to confer a particular interpretation of these signals in a specific manner: tooth, limb, heart or liver.

As a group of cells develop into a particular structure, something has to keep the 'group identity' constant. The wing of *Drosophila* provides a useful system to unravel the molecular basis of this notion of identity. Developmental studies have shown that the *vestigial* (*vg*) gene encodes a nuclear protein that plays a central role in the development of the wings in *Drosophila*: loss of *vg* results in the failure of wings to develop (Lindsley and Zimm, 1992) and ectopic expression of *vg* leads to the development of ectopic wing tissue (Kim et al., 1996). These experiments suggest that *vestigial* can be viewed as a 'wing selector' gene i.e. a gene that enforces wing developmental pathway on cells (Kim et al., 1996).This notion is supported by the observation that ectopic expression of vestigial can rescue the loss of wing caused by mutations in genes involved in the regulation of wing development such as *apterous*, *Suppressor of Hairless* or *wingless* (Klein and Martinez Arias, 1998a).

The expression of *vg* during wing development is regulated through two enhancers called the second intron or boundary enhancer (*vg*BE), and the quadrant enhancer (*vg*QE) (Williams et al., 1994; Kim et al., 1996; and Fig. 1). These names reflect the patterns of expression directed by these regulatory regions: the vestigial Boundary Enhancer (*vg*BE), a thin stripe over the prospective wing margin, and vestigial quadrant enhancer (*vg*QE), a pattern in four quadrants that are complementary to the *vg*BE and which fill in the developing wing blade. Both, *vg*BE and *vg*QE, act as integrators of signalling systems that drive wing development and, in this manner, these regulatory regions determine the tempo and the mode of wing development (Williams et al., 1994; Kim et al., 1996).

However, the function of *vg* and, more specifically, how it endows cells with identity is not clear. One possibility is that Vestigial regulates patterns of proliferation and gene expression that are characteristic of wing tissue. However, there is no direct evidence for this.

Here we study the regulation of the *vg* gene. We show that Vestigial is required for its own expression and that the three signalling systems known to play a major role in wing development, Notch, Wingless and decapentaplegic (*dpp*), not only act through the known enhancers of *vg*, but also show synergistic interactions with the *vg* gene product. These observations lead us to suggest that vestigial specifies identity in part by providing a molecular context in which different signalling events act to trigger the development of a wing. Perhaps by responding to this signalling system, Vestigial targets factors onto specific genes required for the proliferation and patterning of the wing. Our results further show that *vg* plays an important role together with, rather than simply downstream of, *wingless* in the patterning of the wing blade along the dorsoventral axis. The experiments suggest that the main function of *wg* is to enforce gene expression rather than initiate it.

MATERIALS AND METHODS

Drosophila stocks

The *Ser* mutants used in this study are Ser^{RX106} (Speicher et al., 1994) and Ser^{94C} (Couso et al., 1995). Expression of *vg* at the DV boundary was detected using the vestigial Boundary Enhancer described in Williams et al. (1994) and referred to here as *vg*BE (for Boundary enhancer). The *vg*-quadrant enhancer is described in Kim et al. (1996, 1997b) and is referred to here as *vg*QE. For the expression of *wg* in the developing discs, we sometimes used a P-*lacZ* insertion in the *wg* gene on a CyO chromosome (Kassis et al., 1992).

Lines allowing us to overexpress the dominant negative UAS-*wg* carry a *wingless* gene with a stop codon at codon 398 of the *wingless* coding region (Zecchini and A. M. A., unpublished data). This C-terminal deletion mimics a mutation in *Xenopus* Wnt (Hoppler et al., 1996) and causes dominant negative effects in the embryo and the adult; details of the construct and the flies themselves are available upon request.

Ectopic expression of the different genes was achieved through the GAL4/UAS system of Brand and Perrimon (1993). The UAS-*vg* constructs were kindly provided by Sean Carroll (Kim et al., 1996); UAS-*wg* by K. Gieseler and J. Pradel; UAS-*Nintra* and UAS-*Dl* by L. Seugnet and M. Haenlin. The UAS-GFP is described in Yeh et al. (1995).

The expression of the different UAS constructs was driven in the imaginal discs with various GAL4 inserts. *patched* Gal4 (*ptc* GAL4) expresses UAS-*X* in a stripe along the AP boundary of the discs (Speicher et al., 1994). In the third instar, *decapentaplegic* Gal4 (*dpp* GAL4) (Wilder and Perrimon, 1995) is expressed in a similar pattern to *ptc* GAL4, although the expression is weaker over the ventral side (Klein and Martinez Arias, 1998a). The *vg*BEGal4 is described in Neuman and Cohen (1996a).

Stocks carrying various GAL4 and UAS combinations in wild type and mutants were generated. All stocks were balanced over the SM6a-TM6b compound balancer, which allowed the identification of larvae of the correct genotype because of the dominant larval marker Tb (Lindsley and Zimm, 1992). Details of the stocks as well as the stocks themselves are available upon request. In the case of *ap* mutations, stocks were established with a CyO balancer carrying a P-*lacZ* insertion in *wg* and the mutant discs were checked for the absence of the *wg* expression pattern. In the case of some experiments involving *ap* mutants, mutant discs were identified by the aberrant morphology of the wing disc and the absence of the CyO *wg-lacZ* balancer.

Immunohistochemistry and in situ hybridization

The Distalless antibody was a gift from S. Cohen and S. Bray and stainings were performed according to standard protocols. In situ hybridizations were performed as described in Tautz and Pfeifle (1989). The X-Gal staining is described in Ashburner (1989). The fluorescence of the green fluorescent protein (GFP) was detected by using the FITC-filter sets on a Zeiss Axiophot microscope.

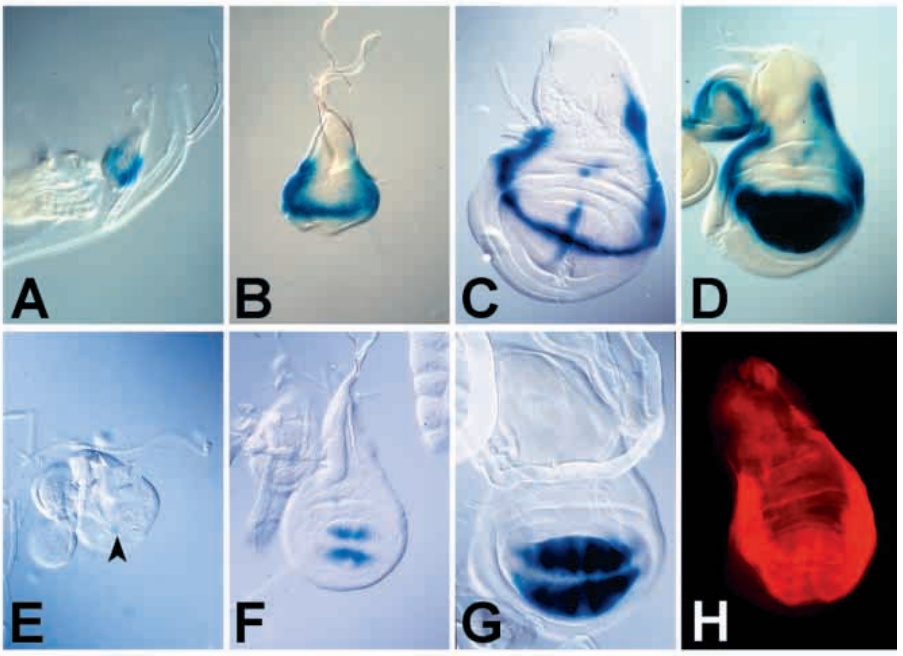

Fig. 1. Expression of *lacZ* directed by the vestigial boundary enhancer, *vg*BE (A-C), the vestigial quadrant enhancer, *vg*QE (E-G), both together (D) and the endogenous Vestigial protein (H), throughout wing development. All discs shown with anterior to the left and ventral to the bottom. (A) Activity of the *vg*BE is detected in the second instar in a horseshoe pattern, with weaker expression in a region that fate maps to the anterior ventral region of the disc. (B) At the beginning of the third instar, the *vg*BE is active in a stripe that fate maps the wing margin and extends on either side of the wing disc into the flanks of the notum. At this stage, the wing primordium is the domain of the *vg*BE in the center of the disc. (C) The pattern defined at the beginning of the third instar is maintained throughout this stage, with the addition of a stripe along the AP boundary. (D) The patterns of the *vg*BE and *vg*QE, together, make up for the pattern of *vg* expression (compare with H) during wing development (Kim et al., 1996). (E) The *vg*QE is not active in the second instar, but becomes activated at the beginning of the third instar in a region around the intersection of the AP and DV boundaries (arrowhead). (F,G) Throughout later stages of the third instar, the activity of the *vg*QE outlines the developing wing pouch, is excluded from the wing margin and is lower in the regions between the proteins. (H) Pattern of Vestigial protein expression in a third instar wing disc. Comparison with C, D and G indicates that *vg*BE and *vg*QE account for the complete pattern of *vg* expression, except in the adepithelial cells underneath the notal region.

RESULTS

The expression of *vg* during wing development is regulated by two enhancer sequences located in the second and fourth introns of the gene (Williams et al., 1994; Kim et al., 1996). The second intron enhancer regulates expression of *vg* along the DV boundary (Williams et al., 1994) and is therefore called the boundary enhancer (*vg*BE). The enhancer located in the fourth intron is called quadrant enhancer (*vg*QE), is activated much later than the *vg*BE (see Fig. 1 and Kim et al., 1996) and regulates the expression of *vg* in the pouch away from the DV boundary. This enhancer is only active in the wing pouch of the wing disc and not in any other imaginal disc.

The development and patterning of the wing depends on a series of interdependent regulatory loops that define a sequence of spatial patterns of gene expression. For this reason, it is important to determine both spatial and temporal aspects of the inputs on different genes (Klein and Martinez-Arias, 1998a). We do this below by focusing on the regulation and function of the two main enhancers of the *vg* gene.

Early activity of the vestigial DV boundary enhancer (*vg*BE)

The *vg*BE is activated first during the second instar and its expression pattern is very similar to that of the Vestigial protein at this stage (Klein and Martinez Arias, 1998b) suggesting that, in these early stages, the *vg*BE is responsible for the complete pattern of *vg* expression (also see below). Deletion analysis of the enhancer reveals two regions essential for its activity: a binding site for Suppressor of Hairless and sequences contained in the first 80 base pairs of the enhancer (Williams et al., 1994; Kim et al., 1996, 1997b).

In an attempt to map the nature and timing of the inputs into this enhancer, we have compared during wing development the activity of the wild-type *vg*BE and of deletions of the two essential regions (Fig. 2). To do this, we used *lacZ* fusions and monitored their expression in the developing wing disc (Williams et al., 1994; Kim et al., 1996, 1997b). We find that at the end of the second instar, *lacZ* expression from the wild-type *vg*BE outlines a horseshoe over the ventral region of the wing disc, with weak expression in the ventral anterior region where it overlaps with the expression of *wingless* (Figs 1A, 2A,L). *lacZ* expression increases in this region at the beginning of the third instar (Figs 1B, 2B,C). This increase does not occur in *Su(H)* mutants (data not shown), or in *wg* mutants (Klein and Martinez Arias, 1998a).

The activity of the enhancer deleted for the *Su(H)*-binding

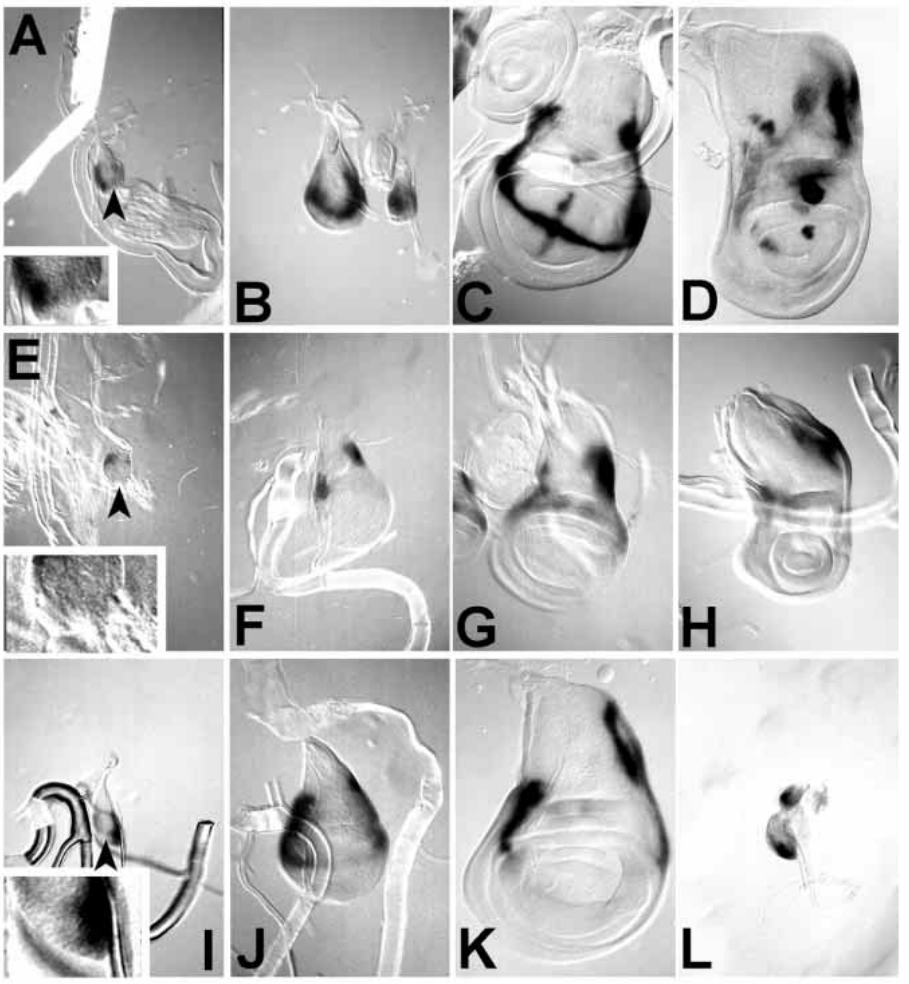

Fig. 2. Expression of *lacZ* directed by the wild-type and mutated *vg*BE in wild-type (A-C,E-G,I-L) and *Su(H)* mutant wing discs (D,H). All discs shown with anterior to the left and ventral to the bottom. (Inserts in A,E and I) Higher magnifications of the ventral region where the wing primordium is established. (A-C) Pattern of activity of the *vg*BE in wild-type wing discs of second (A), early third (B) and late third (C) instar. (D) In *Su(H)* mutant discs, the activity of the *vg*BE is abolished from the wing region and most of the notum. (E-G) Activity of a *vg*BE deleted for the *Su(H)* binding site. (E) In the second instar, there is no activity over the center of the disc (arrowhead; compare with A and see insert). Also during later stages no activity was detected in the region of the developing wing (F,G). Notice that, nevertheless, there is activity of the enhancer at the flanking regions of the wing primordium from early on. (H) Expression of *lacZ* directed by the *vg*BE deleted for the first 80 bp in a *Su(H)* mutant. The residual notal expression indicates that there are other inputs in this enhancer. (I-K) Expression of *lacZ* directed by the *vg*BE deleted for the first 80 bp in the wild type. (I) In the second instar, the activity of the deleted *vg*BE is similar to that of the wild-type one (see insert; compare with A). However, as the wing grows, there is no enhancement over the developing wing pouch and faint activity can be detected over the DV boundary as the disc grows (J) and eventually disappears (K). Very faint expression can sometimes be observed, perhaps due to perdurance of the *lacZ* (K). (L) Coexpression of *lacZ* driven by the *vg*BE lacking the first 80 bp and *wg* in a second instar disc. The region of low activity is covered by the expression of *wg* (compare with I). Since *wg* expression defines the region of the wing primordium, the double staining reveals that the region of low expression is in the region of the developing wing. Arrowheads in A, E and I are indicating the region where the wing primordium develops.

site is different from the wild type. The activity of this enhancer is never initiated over the ventral region of the disc, where the wing primordium is established and remains absent during later stages (Fig. 2E-G). This result demonstrates that the activity of *Notch* is required not only for the maintenance, but also for the initiation of the expression of vg through the *vg*BE (Klein and Martinez-Arias, 1998a; Fig. 2).

The activity of the enhancer deleted from 0-80 is similar to that of the wild-type enhancer early on (Fig. 2I), but it never acquires the high levels of activity in the anterior ventral region. As the wing blade develops, a line of faint activity can be detected over the DV boundary (Fig. 2J), but it fades quickly and, by the end of the third instar, in most discs there is no activity over the developing wing blade (Fig. 2K). This enhancer still shows some activity in the flanking notal regions of the wing disc in wild-type and *Su(H)* mutant discs (Fig. 2H,K). This indicates the existence of additional inputs in the regulation of the *vg*BE in the notal region (see also Kim et al., 1997a).

Our results (and see also Klein and Martinez Arias, 1998a) suggest that the cells in which the expression of the *vg*BE is upregulated at the end of the second instar represent the anlage of the wing and require *Notch*/*Su(H)* signalling. These cells are located at the DV interface, on the domain of *wg* expression and overlap the expression on *nubbin*. Our suggestion that these cells represent the primordium of the wing pouch can explain why a deletion of the *vg*BE, as in the vg^{83b27} allele, results in the abolition of the development of the wing pouch since, in this mutant, the anlage would never be defined.

Wingless is an input on the DV Boundary Enhancer (*vg*BE)

We have recently demonstrated that, in addition to the activity of Notch, the activity of *wg* is required for the initiation of *vg* expression since, in second instar larval discs of *wg* mutants, the activity of the *vg*BE is absent over the region of the wing anlage (Klein and Martinez Arias, 1998a). The possibility that Wingless acts on the vgBE is supported by the observation that a dominant negative version of DFrizzled2, a receptor for Wingless, reduces the activity of the *vg*BE (Zhang and Carthew, 1998). Altogether these results raise the possibility that Wingless has a direct input on the *vg*BE. Consistent with this we find that the first 80 bp of the *vg*BE, which are required for the full activity of the enhancer, contain two putative TCF-1-binding sites that are associated with Wingless signalling (Fig. 3A,B).

To test further the relationship between *wingless* and *vg* early in wing development, we monitored the influence of Notch and Wingless signalling on the activity of the *vg*BE. In contrast with ectopic expression of *wg*, which never leads to ectopic expression of the *vg*BE (Fig. 3C), ectopic expression of *Delta* with *ptc*GAL4 leads to ectopic expression of the *vg*BE, but only within the developing wing blade and where the levels of ectopic expression of *Delta* are high, near the AP compartment boundary (Fig. 3D). Interestingly, an effect of Wingless on this enhancer can be detected when Wingless is coexpressed ectopically with Delta. Although Wingless alone has no effect on this enhancer, coexpression of Delta with Wingless extends the realm of activity of the *vg*BE into regions where expression of Delta on its own has no detectable effects regions (Fig. 3E and see also Klein and Martinez Arias, 1998a).

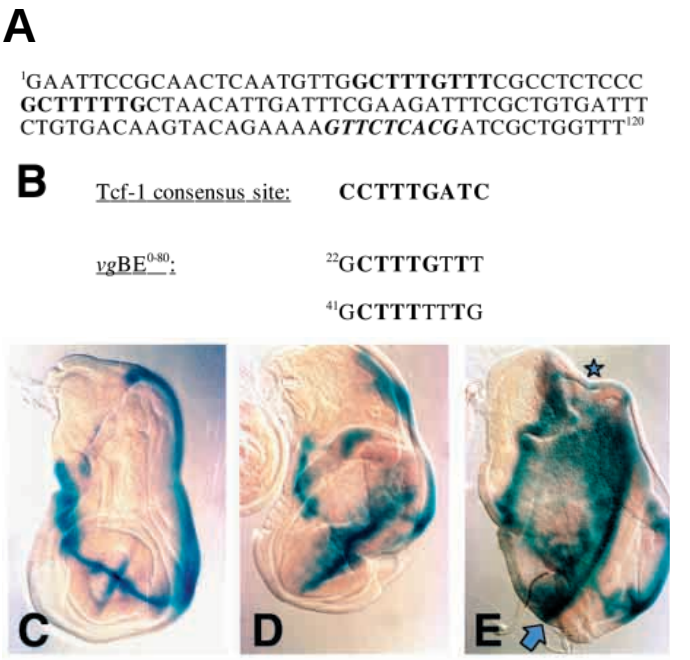

Fig. 3. Influence of *wg* and *N* signalling on the *vg*BE. (A) The first 120 bp of the *vg*BE with the putative Tcf-1 sites highlighted in bold and the *Su(H)* binding site highlighted in italics. (B) Comparisons of the two sites found in the first 80 bp of the *vg*BE with the Tcf-1 consensus binding site. (C) Expression of UAS-*wg* with dppGal4 does not change the activity of the *vg*BE, although morphological changes are visible in the wing disc, which are the result of the ectopic induction of hinge fate in the notal area (Klein and Martinez-Arias, 1998a; T. K. and A. M. A., unpublished data). (D) Expression of UAS-*Dl* is ectopically inducing the activity of the *vg*BE in the wing pouch perpendicular to the DV boundary. (E) The coexpression of UAS-*wg* and UAS-*Dl* results in a stronger activity of the enhancer in the pouch and in the extension of the ectopic expression domain into notal area (asterisk). Further, as we have reported before, the coexpression of the two genes leads to the extension of the blade into the peripodial membrane and the expression of the *vg*BE is also extended into this region. The arrow indicates the point where the pouch is extending into the region of the peripodial membrane. Since the peripodial membrane is covering the disc, the extension comes to lie underneath the normal pouch area and. consequently, the extension of the expression of the *vg*BE is not visible in this photograph (see Klein and Martinez Arias, 1998a and Fig. 5E for further details).

These results suggest that the major effect of Wingless on the *vg*BE is to collaborate with Notch/Su(H) signalling during the early stages of wing development. The failure to observe effects of ectopic expression of Wingless on this enhancer might be related to the absolute requirement for Notch signalling in this function of Wingless. Since, during the early stages of wing development, Notch signalling is restricted to the DV interface (see Klein and Martinez Arias, 1998b), the effects of Wingless could only be observed in this region and are highlighted by the loss of *wg* function or the loss of Wingless responding sites in the enhancer. Extension of this domain by activating Notch signalling ectopically allows the visualization of the effects of ectopic Wingless. It is likely that the residual activity of the *vg*BE$^{\Delta 0\text{-}80}$ observed along the DV boundary during early stages of wing development reflects the

input of Notch signalling without the enhancement of Wingless signalling.

Notch signalling is required for the initial activity of the Quadrant Enhancer (*vg*QE)

The activity of the *vg*QE can be detected first at the beginning of the third instar, several hours after the upregulation of the *vg*BE, when it closely outlines the realm of the growing wing (Fig. 1E-H and see Kim et al., 1996). This enhancer is only expressed in the growing wing blade and thus provides a unique and most specific marker for wing blade tissue.

A variety of experiments have shown that the *vg*QE receives a negative input from *Notch* signalling and a positive one from Dpp (Kim et al., 1996 and see below). The presence of a *E(spl)*-binding site in the sequence of the *vg*QE led to the suggestion that this suppression by *Notch* is mediated by the *E(spl)* protein. However, we do not find a strong suppression of the activity of the *vg*QE if UAS-m8 (*E(spl)*) is ectopically expressed with *dpp*Gal4 (data not shown), suggesting that the effect of *Notch* requires other mediators. In our experiments, we find that, although the *vg*QE is suppressed in the domain of *Notch* activity (Fig. 5D), *Notch* signalling plays a non-autonomous role in its activation. For example, the *vg*QE is never active in *Serrate* (*Ser*) mutants in which wing development initiates normally but is aborted very early (Klein and Martinez-Arias, 1998b; Fig. 4A). Expression of UAS-*Dl* under the control of *dpp*Gal4 in *Ser* mutants rescues the wing pouch and leads to the activation of the *vg*QE (Fig. 4B). Interestingly, this activity arises in regions devoid of *Notch* signalling (compare Fig. 4B and C). This result suggests that *Notch* signalling influences the activity of the *vg*QE in two ways: it represses the activity of the *vg*QE autonomously but it is also required for its activity in a non-autonomous way.

Wingless acts synergistically with Vestigial to promote the activity of the Quadrant Enhancer (*vg*QE)

It is clear that the activity of the *vg*BE is required for the activation of the *vg*QE (Kim et al., 1996; T. K. and M. A. M. unpublished data), but little is known about how this interaction takes place. The observation that *vg*QE is activated in *Ser* mutant discs in which UAS-*vg* is expressed ectopically suggests that the activation of the vgQE is mediated by *Notch* signalling through the activity of Vestigial on the *vg*QE (Fig. 4E,F). However, in this experiment, expression of *wg* is also restored (Fig. 4F) and this raises the possibility that activation of the *vg*QE is mediated through the presumed 'organizing' activity of Wingless (Zecca et al., 1996; Neumann and Cohen, 1996b). This is probably not the case since we find that ectopic expression of UAS-*wg* alone does not lead to the activation of the *vg*QE in *Ser* mutants (Fig. 4D). The inability of Wingless to activate the *vg*QE in this experiment is not due to a general insensitivity of the cells to Wingless signalling, since UAS-wg is always capable of inducing hinge fate ectopically (Klein and Martinez-Arias, 1998a).

These results clearly demonstrate a requirement for *vg* in the activation of the *vg*QE. However, clonal analysis has shown that *vg* acts cell autonomously (Kim et al., 1996) and therefore, in the wild type, the non-autonomous effects of *Notch* on the *vg*QE must be mediated by another, diffusible molecule(s) which is under control of Notch signalling. A number of studies suggest that Wingless has an influence on the expression of *vg* in the wing pouch and that its expression at the wing margin is under control of *Notch* signalling (Zecca et al., 1996; Neumann and Cohen, 1996b). Therefore, it is possible that Wingless is mediating the non-autonomous effect of *Notch* on the *vg*QE. It might be that Wingless acts by acting on the *vg*QE to elevate and maintain the levels of *vg* expression that had been induced by Vestigial through the *vg*BE. In agreement with this proposal, we find that the activity of the *vg*QE is elevated in response to the ectopic expression of UAS-*wg* with dppGal4 (Fig. 5C,H) and that expression of a dominant

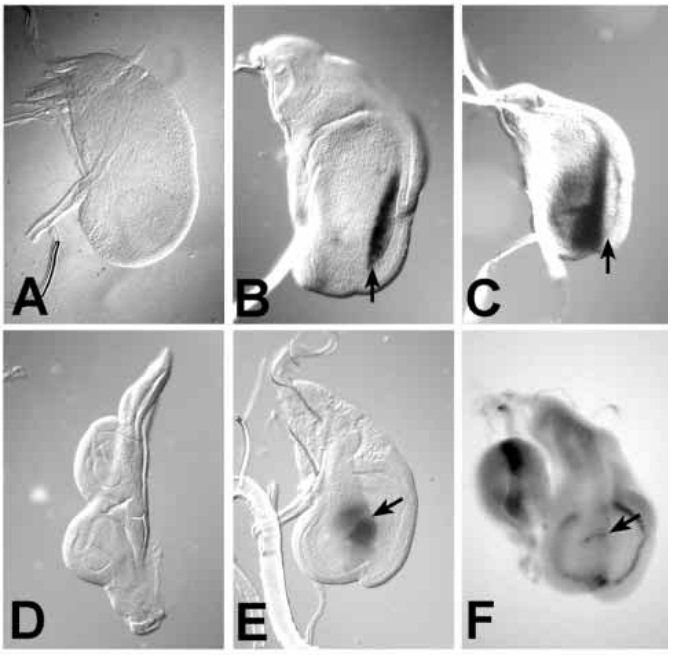

Fig. 4. *Notch* activity is required for the activation of the *vg*QE. (A) In a Ser^{94C}/Ser^{RX106} mutant wing disc, the *vg*QE is never activated. (B) The activation of the *Notch*/*Su(H)* pathway in the Ser^{94C}/Ser^{RX106} wing disc by the expression of UAS*Dl* with *dpp*Gal4 rescues the loss of wing tissue and leads to the activation the *vg*QE in the posterior region of the recovered pouch (arrow). (C) Detection of *Notch* activity in Ser^{94C}/Ser^{RX106} wing disc in which UAS*Dl* is expressed by *dpp*GAL4 by monitoring the expression of the target gene *wg*. Arrow indicates the region corresponding to the same region in B. The comparison of the expression of *wg* and that of the *vg*QE suggests that the *vg*QE is induced non-autonomously by the ectopic expression of *Dl*. The results also demonstrate the requirement of *Notch* activity for the activation of the *vg*QE. (D) Expression of UAS-*wg* by dppGal4 does not activate the *vg*QE. (E) However, expression of UAS-*vg* in the same genetic background does activate the *vg*QE. A clear stripe of suppression of the activity of the *vg*QE is observed in the region of the DV boundary (arrow) suggesting that *N* signalling occurs in this region. This is further supported by the fact that a small stripe of *wg* expression is detectable until mid/late third instar (arrow). The expression of *wg* along the DV boundary is normally absent in this Ser mutant combination and suggests that the provided activity of *vg* leads to the enhancement of the residual *N* activity detectable in Ser mutant wing discs during early wing development (Klein and Martinez Arias, 1998b). Indeed, it has been previously shown that *vg* is collaborating with *Notch* signalling to activate *wg* expression along the DV boundary during normal wing development (Klein and Martinez Arias, 1998a). For further information see text.

negative Wingless molecule (see Materials and Methods) suppresses the activity of the *vg*QE and reduces the size of the wing pouch (Fig. 5J).

Altogether our results suggest that the upregulation of *vg* expression in response to *wg* is mediated by the *vg*QE. Our conclusion is supported by the existence of several putative TCF-1 binding sites in the *vg*QE (data not shown). However, the effects of Wingless are always restricted to the normal domain of *vg* expression (compare Fig. 3F,K), in agreement with the results presented above that Wingless alone is not sufficient to initiate ectopic expression of *vg* through the *vg*QE. These effects are likely to be mediated by Vestigial itself and, as indicated above, the role of Wingless on this regulation is to maintain and modulate the levels of activity of the *vg*QE. To test this possibility, we have expressed *vg* under the control of *dpp*GAL4 and observed that this leads to elevated activity of the *vg*QE and, in addition to weak, but reproducible amounts of ectopic activity of the *vg*QE in the notal region (arrows in Fig. 5F). No ectopic expression of *wg* is observed in the dorsal side of the wing disc in these experiments and therefore the ectopic activation of the *vg*QE in the dorsal is probably independent of Wingless signalling (Fig. 5L). Although this does not rule out the existence of regulatory intermediaries, it does suggest that Vestigial has an effect on the activity of this enhancer.

Consistent with our conclusion that Wingless enhances the effects of Vestigial, we find that coexpression of Wingless and Vestigial with *dpp*GAL4, which leads to the ectopic induction of pouch and hinge fate in the notal regions (Klein and Martinez-Arias, 1998a), triggers a widespread and stable expression of the *vg*QE throughout the wing disc (Fig. 5G). This synergistic effect is more clear in the case of the leg discs where the *vg*QE enhancer is not active during normal development. Expression of *wg* alone in the leg disc does not activate the *vg*QE and, similar to the wing disc, *vg* alone leads to some weak activation of this enhancer (data not shown). However, their combined expression leads to very robust and widespread expression of this enhancer (Fig. 5M).

These results suggest that the activity of *wg* is required to enhance the activity of the *vg*QE which is initiated by the Vg protein itself. They further suggest that the role of Notch in the regulation on the activity of the *vg*QE is twofold. First, it activates the expression of Vestigial (via the *vg*BE) and of *wg* along the DV boundary. This sets up the conditions for the activation of the *vg*QE through Wingless and Vestigial as the wing pouch begins to grow. Second, at the DV boundary, *Notch* signalling leads to a suppression of the activity of the *vg*QE but maintains the expression of *wg* and of the *vg*BE.

To confirm this conclusion, we have monitored the influence of coactivation of Wingless and Notch signalling on the activity of the *vg*QE enhancer. We have shown previously that the definition of the size and the fate of the wing pouch requires the combination of Wingless and Notch signalling on the *vg*BE (Klein and Martinez-Arias, 1998a and this work). We now find that ectopic expression of *Dl* or *wg* alone are not sufficient to activate the *vg*QE (Fig. 5C,D; see above) but that the coexpression of both results in the extension of the *vg*QE into the notum as it has been previously shown for *vg* expression by antibody staining (Fig. 5E; Klein and Martinez-Arias, 1998a).

The interactions between Dpp and Wingless during wing development take place at the level of the *vg*QE and require the *vg* gene product

Multiple experiments have shown that *dpp* provides an important input into the growth of the wing (see e.g. Zecca et al., 1995) but appears not to play a role in the initiation of wing development since wings derived from *dpp* mutants that affect its function during wing development, have a normal margin (see for example Zecca et al., 1995). However, the *vg*QE contains functional Dpp response sites (Kim et al., 1997a) and responds to Dpp (Kim et al., 1996). These observations, together with those of other inputs on the expression of *vg*, have led to the suggestion that, as the wing grows, it integrates inputs from both *wg* and *dpp* (Kim et al., 1997a).

To test this integration directly, we have created a complete overlap of the activities of *wg*, *dpp* and *vg* by activating UAS-*wg* and UAS-*dpp* with *vg*BEGAL4 (Staehling-Hampton and Hoffmann, 1994; Neumann and Cohen 1996a and Fig. 6A). Ectopic expression of *wg* alone in this manner leads to ectopic expression of the *vg*QE in the posterior region of the domain of *vg*BE expression (Fig. 6B), over a region in which *dpp* is normally expressed (compare Fig. 6B with A and G). Interestingly, elevation of *wg* expression achieved in the area of the DV boundary does not enlarge the wing pouch (compare Fig. 1G and 6B). This suggests that, although *wg* is required for the correct growth of the pouch, it is not a limiting factor. Expression of *dpp* under the same conditions leads to an enlargement of the wing blade along the AP axis, with the *vg*BE as a reference but without extending it into the notum (Fig. 6C).

Coexpression of *wg* and *dpp* leads to a pronounced extension of the wing blade towards the notum (Fig. 6D). In some instances, we can observe the development of up to eight wing blades, all with a common origin over a point ventral to the notum (Fig. 6E,F). All these wings express the *vg*QE and each of them has a defined margin (Fig. 6F). It is possible that these multiplets are produced by splitting of an initial primordium. It is the case that the expression driven by *vg*BEGAL4 is discontinuous (see Fig. 6A) and therefore leads to the generation of several foci of overlap of the activities of *wg* and *dpp*, which appear to act as foci for growth and patterning.

In the wild type, the activity of the *vg*QE is initiated at the intersection of *wg* and *dpp* expression and radiates from this focus (see Fig. 1 and Kim et al., 1996). The results presented here indicate that this overlap determines important parameters of the morphogenesis of the wing. For example, it is possible that the distance from this focus – perhaps defined by the range of diffusion of the two molecules – defines a threshold that contributes significantly to the determination of the size and shape of the wing blade. By tampering with these overlaps, one can alter the shape of the wing (Fig. 6) or, by generating a series of them, trigger the development of multiple wings from one primordium.

Vestigial contributes to the differential patterns of gene expression in the wing blade

A variety of experiments have suggested that the expression of *wg* at the wing margin is a source of Wingless which, in a concentration-dependent manner, determines patterns of gene expression (Zecca et al., 1996; Neumann and Cohen, 1996b). One of these targets is the *vg* gene and here we have shown

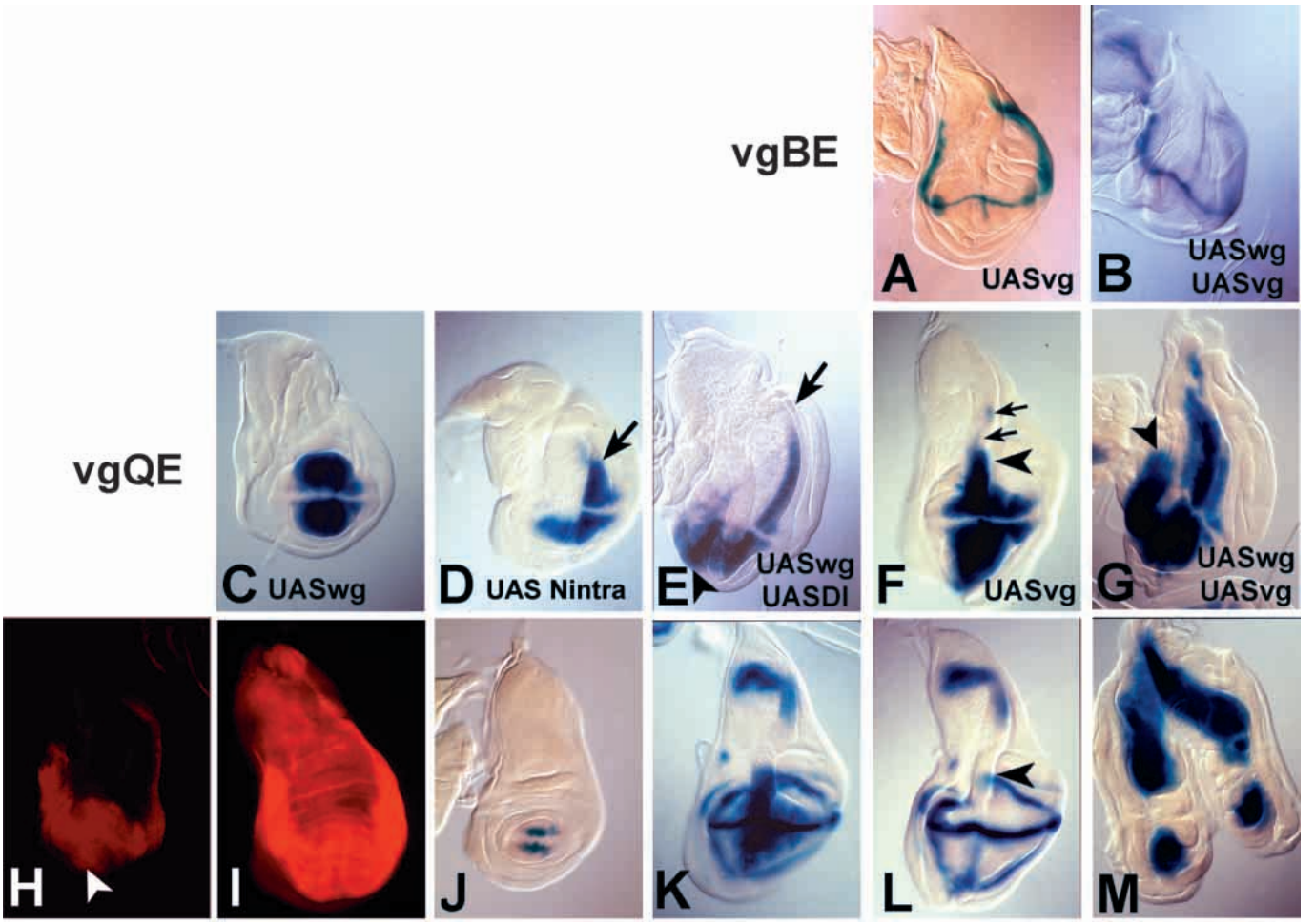

Fig. 5. Activity of the *vg*BE (A,B), the *vg*QE (C-G,I,J,M) and *wglacZ* (K,L) as a response to various inputs during wing development. In all cases, except J, ectopic expression was elicited with *dpp*GAL4, which directs gene expression along the AP boundary, with higher levels on the dorsal than on the ventral sides (see Fig. 6G). In J, the expression of UAS-wg^{-C} was expressed with sdGal4, which activates expression in the area of the wing throughout wing development (A). Expression of *vg* has no effect on the activity of the *vg*BE and this is not altered even by coexpression of *vg* with *wg* (shown in B). (C) Expression of *wg* leads to an upregulation of the activity of the *vg*QE within its normal realm of expression (compare with anti-Vestigial staining in I and Fig. 1F). (D) Activation of *Notch*/*Su(H)* signalling with UAS-Nintra leads to a suppression of the activity of the *vg*QE within the anterior region of the disc, which is where *Notch* signalling is being ectopically activated. The effects are restricted to the normal domain of the wing pouch, as indicated by the arrow. The effect is weaker over the ventral region of the disc, where the expression of *dpp*GAL4 is weakest (see Fig. 6G). (E) Coexpression of *Dl* and *wg* has two different effects on the activity of the *vg*QE. Over the anterior region, it has an effect similar to Nintra and suppresses its activity (compare with D); however, over the posterior region, there is a large region of ectopic expression that reaches into the notum (arrow) and into the above lying peripodial membrane (arrowhead). Since *dpp*GAL4 is not expressed over this region, this effect is non-autonomous and must be due to the activity of *wg*. Furthermore, because on their own expression of *Dl* or *wg* never produces this effect, this observation highlights the synergism of their combined activities (see also Klein and Martinez-Arias, 1998a for further details concerning the expansion of the wing pouch into the notal region in response to the coexpression of the two genes). (F) Expression of *vg* leads to ectopic expression of *vg*QE not only over the hinge region, but also extending into the notal region (small arrows). The ectopic expression of *vg* does not lead to ectopic activation of *wg* (see L) and therefore the effects of *vg* on the *vg*QE are probably not mediated by *wg*. (G) Coexpression of *vg* and *wg* leads to a strong enhancement of the ectopic activity of the *vg*QE showing that *wg* can enhance the induction of the *vg*QE by *vg*. Notice that ectopic expression of *wg* alone is not able to elicit ectopic induction of the activity of the *vg*QE (see C). (H) Expression of Vestigial protein after expression of *wg* under the control of *dpp*GAL4. Notice the elevated levels of expression along the AP boundary (arrowhead). (I) Wild-type expression of Vestigial revealed by antibody staining, for comparison with H. (J) Expression of a dominant negative form of wg, UAS-wg^{-C}, results in the reduction of the wing pouch and the reduction of the activity of the *vg*QE. (K) Expression of *wg* (*wglacZ*) and *vg*QE, after expression of *vg* under the control of *dpp*GAL4. The expression of *wg* in the hinge region indicate the limits of the wing area and indicates that high levels of activity of the *vg*QE are restricted to the wing region. The extension of the activity of the *vg*QE into hinge domains indicates that *vg* transforms hinge into wing blade. (L) Expression of *wg* (*wglacZ*) after expression of *vg*. Notice that there is almost no effect on *wg* expression other than the disruption of the normal pattern of expression around the hinge. This effect is likely to be due to the *vg*-mediated transformation of hinge into blade and shows that the effects of *vg* on the expression of the *vg*QE, shown in F,K, are independent of *wg*. (M) Coexpression of *wg* and *vg* leads to high levels of activity of the *vg*QE in the leg discs. On its own, the expression of *vg* leads only to weak ectopic activation of the *vg*QE in the leg discs (data not shown) and we have never found any ectopic activity of the *vg*QE if *wg* is expressed in the leg disc. The *vg*QE is not active in the leg discs during normal development. This suggests that, similar to the situation in the wing disc, *wg* can enhance the induction of *vg* expression by Vestigial protein but not initiate *vg* (see text).

that, in the wing blade, these effects are likely to be targeted through the *vg*QE. However, here we have shown that Vestigial can alter the spatial activity of the *vg*QE without altering the endogenous pattern of *wg* expression. Since *vg* is itself expressed in a gradient similar to that generated by the diffusion of Wingless (Williams et al., 1991; Zecca et al., 1996; Neumann and Cohen et al., 1996), and displays synergistic interactions with Wingless (Figs 2, 3), this raises the possibility that Vestigial operates together with, rather than downstream of, Wingless in the regulation of gene expression within the wing pouch. To test this, we have studied the effects of Vestigial on another target of Wingless signalling: *Distalless* (*Dll*).

In the wild-type wing, *Dll* is expressed in the wing pouch in a circle of cells that lies within that defined by the expression of *vestigial* (Zecca et al., 1996; Neumann and Cohen, 1996b and Fig. 7A). We find that expression of *vg* with *dpp*GAL4 elevates the levels of *Dll* expression within the developing wing blade and triggers ectopic expression of *Dll* outside this area (Fig. 7B). This differs from the effects of ectopic expression of *wg*, which are always restricted to the developing wing blade and never induce ectopic expression of *Dll* (Zecca et al., 1996; Neumann and Cohen, 1996b and Fig. 7C). Coexpression of both, *wg* and *vg*, results in synergistic effects similar to those described before for other targets and an increase in the area of ectopic expression (Fig. 7D).

The effects of *vg* are independent of *wg* (Fig. 7E), since they are achieved even in *ap* mutant where *wg* is never expressed along the DV boundary (Fig. 7F-H and see also Ng et al., 1996). In these mutants, there is no *Notch*/*Su(H)* signalling, but ectopic expression of *vg* can rescue the loss of the wing pouch caused by the loss of *ap* function (Klein and Martinez Arias, 1998a). Although *vg* is required for *wg* expression, there is not much *wg* expression in the rescued *ap* mutants. However, there is a clear stripe of *Dll* expression that correlates with the expression of *vg*.

These results, together with our previous observation that Vestigial can contribute to the expression of *wg* at the wing margin, indicate that Vestigial can indeed regulate gene expression in the wing blade. In addition, the correlation between levels suggests that different concentrations of Vestigial elicit different effects and that, in some instances, these effects seem to be independent of the levels of Wingless. Thus Vestigial might act in concert with Wingless, and not simply as an effector of *wg*, in the regulation of gene expression.

In our experiments, we notice that very high levels of expression of Vestigial, or long-term exposure to *vestigial* expression, results in the cessation of proliferation, loss of gene expression and, eventually, in cell death (T. K. and A. M. A., unpublished data). This might account for the small of wings that are often visible when *vg* is overexpressed in the developing wing and would correlate with the zone of non-

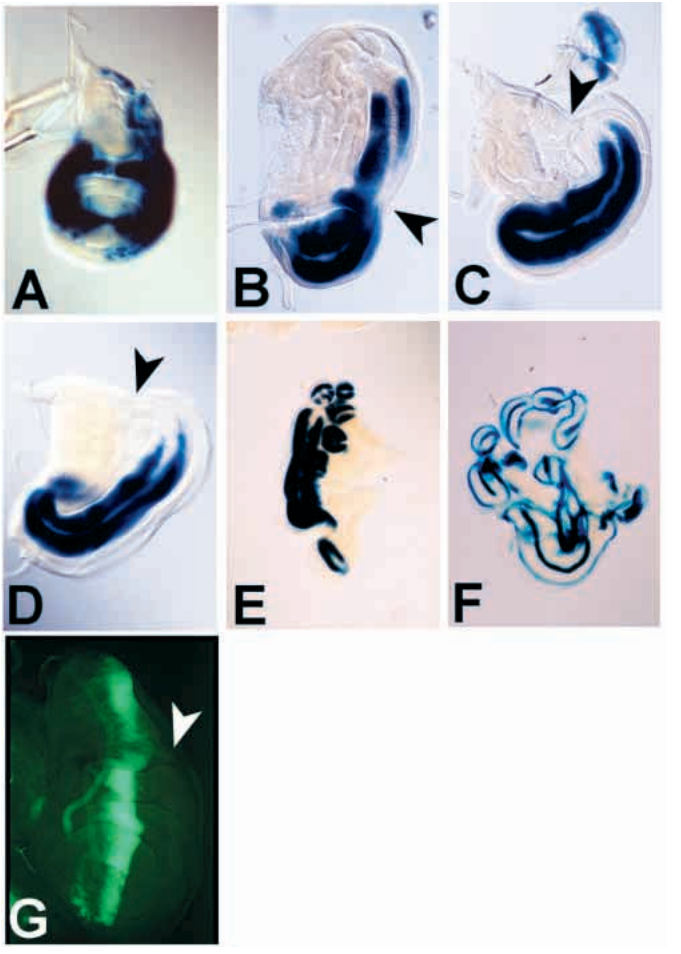

Fig. 6. Effects of the ectopic expression of *wg* (B), *dpp* (C) or both (D-F) under the control of *vg*BEGAL4 on wing development. (A) Pattern of expression of the *vg*BEGAL4 line used in these experiments revealed with UAS-*lacZ*. The pattern is very similar to that of the *vg*BE (compare with Fig. 1C). (B) Activity of the *vg*QE after expression of *wg* in the pattern of the *vg*BE. Notice that there is an extension of the activity of this enhancer over the posterior dorsal region of the disc. This pattern of ectopic activity follows the line of endogenous expression of *dpp* (see G), which overlaps the pattern of expression of the *vg*BE in this region. Arrowhead points to a region where the expression of *vg*BEGAL4 is not overlapping with that of the endogenous *dpp* expression (compare A with arrowhead in G). In this area, the *vg*QE is not activated. If *dpp* is coexpressed with *wg*, the *vg*QE is activated in this region as well (see arrowhead in D). (C) Activity of the *vg*QE after expression of *dpp* in the pattern of the *vg*BE. Notice that there is an extension of the normal pattern along the AP axis, but no change along the DV axis and no extension of the wing into the notum as in the case of ectopic *wg* expression (arrowhead indicates the same position as in B). (D-F) Activity of the *vg*QE in wing discs resulting from the coexpression of *wg* and *dpp* with *vg*BEGAL4. The outcome ranges from the generation of a continuous large wing blade indicated by the continuous expression of the *vg*QE (E,F), to discs where several wing pouches are defined (up to 8) as indicated by the activity of the *vg*QE (E) and *wg-lacZ* expression in F. This 'twinning' effect might reflect the establishment of several primordia early in development and is probably the result of the patchy expression observed in the notal area (see A and text). Each winglet has a margin outlined by the band of no activity of the *vg*QE (in E). These wings indicate that *dpp* can enhance the effects of *wg* and suggests that the overlap of *dpp*, *wg* and *vg* expression is required for the activation of the *vg*QE and the generation of a complete wing during normal development. (G) The expression domain *dpp*GAL4 revealed by the activation of UAS-GFP. GFP is expressed in a stripe along the AP boundary in a AP gradient with the highest levels at the posterior. Arrowhead points to the region where its expression domain does not overlap with that of *vg*BEGAL4.

proliferation that appears at the wing margin, where the levels of *vg* expression are elevated.

DISCUSSION

The *vestigial* gene plays a central role in the development of the wing of *Drosophila*. Previous studies have shown that the spatial and temporal modulation of *vg* expression through specific enhancers is a driving force in the development and patterning of the wing (Williams et al., 1994; Kim et al., 1996, 1997; Neumann and Cohen, 1996a,b; Klein and Martinez Arias, 1998a,b). This regulation is exerted by three major signalling pathways, Notch/Su(H), Wingless and Decapentaplegic, acting differentially on two collections of enhancers: one early acting (*vg*BE) and one later acting (*vg*QE). Here we have shown that the *vg* gene product itself is an important input in the regulation of *vg* gene expression through the *vg*QE and that, in this process, Vestigial acts synergistically with the three signalling pathways that regulate its expression to drive the development of wing blade tissue. This result might provide an answer as to why the activity of the *vg*BE is required for the activation of the *vg*QE. The finding explains also the non-autonomous requirement of the *Notch* signalling cascade, since it is required for the activation of the *vg*BE.

We have also shown that, during early stages of wing development, the expression of *vg* is controlled by the *vg*BE and that Notch signalling is not only required for the maintenance of its activity but also for its initiation. Furthermore, we have provided some evidence to suggest that the activity of Wingless is required for the upregulation of this enhancer. These results add support to, but do not prove, the suggestion that wing development is initiated by the cooperation of Notch and Wingless signalling on the *vg* gene (Couso et al., 1995; Klein and Martinez Arias, 1998a).

The existence of an input of Wingless on the *vg*BE would be consistent with the existence of two putative TCF-1 binding sites in the first 80 bp of this enhancer. Deletion of this region of the *vg*BE substantially reduces its activity over the domain of *wg* expression. Further evidence for a participation of *wg* in the activity of the *vg*BE is provided by the observation that a dominant negative form of the Wingless receptor *Dfrizzled2* leads to a strong reduction of the activity of the *vg*BE (Zhang and Carthew, 1998).

These results contrast with the report that loss of *wingless* function does not affect the activity of the *vg*BE (Neumann and Cohen, 1996a,b). This difference might reflect different ways of carrying out the experiments. The published results are based on clonal analysis, which might not be suitable for the study of the *vg*BE: our results suggest that the loss of *wg* activity reduces dramatically the activity of the enhancer but does not abolish it (Klein and Martinez Arias, 1998 and this work). This means that, taking into account the perdurance of *lacZ* and the limitations imposed by the need of the clones to survive, loss of Wingless in clones might affect the endogenous enhancer but have no observable effect on the reporter. This caveat and our results would account for the observation that large clones of *wingless* mutant cells do not affect the expression of a *vg*BE*lacZ* but display nicks without affecting the size of the wing (see Neumann and Cohen, 1996a): it is likely that in these clones the activity of the endogenous enhancer is affected in a manner that is not reflected by the *lacZ* fusion.

Once *vg* expression is initiated in the wing through the *vg*BE, our results suggest that it is maintained in the growing pouch by a synergistic activity of Wingless and Vestigial on the *vg*QE. This model predicts that loss of the *vg*BE will result in the loss of the wing, and that loss of the *vg*QE should lead to small wings with a well-defined margin. The first prediction is fulfilled by the vg^{83b27} mutation, which removes the *vg*BE

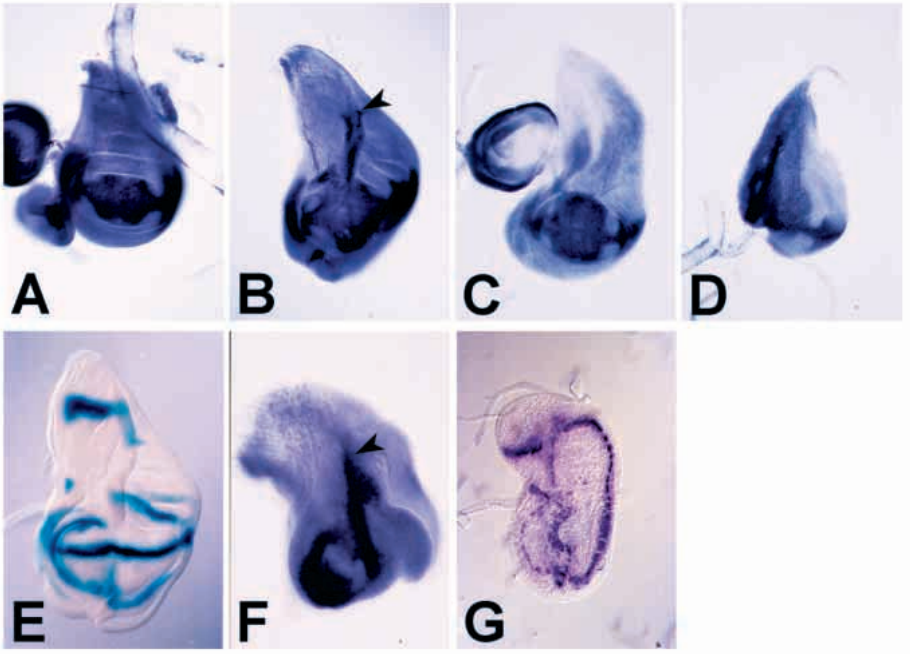

Fig. 7. Effects of the ectopic expression of *vg* on the expression of *Dll* in the developing wing. All experiments make use of *dpp*GAL4 to drive ectopic expression. (A) Pattern of *Dll* expression in a wild-type third instar wing disc as revealed with anti-Dll antibody. (B) Ectopic expression of *vg* leads to ectopic expression of *Dll* in a stripe that overlaps the expression of *vg* and which extends into the notum region of the disc (arrowhead). (C) Ectopic expression of *wg* leads to elevated levels of *Dll* expression within its normal domain of expression, but not to ectopic expression. (D) Coexpression of *vg* and *wg* leads to widespread ectopic expression of *Dll* over the domain of ectopic expression. (E) Ectopic expression of *vg* does not lead to ectopic expression of *wg* instead suppresses the expression of *wg* within the hinge region which is transformed to blade (see Fig. 5K,L). (F) Expression of *vg* in an ap^{UG035} mutant disc rescues the loss of wing pouch tissue of this mutant (Klein and Martinez Arias, 1998a) and leads to ectopic expression of *Dll* over the domain of *vg* expression (arrowhead). (G) Expression of *wg* in a disc as in F, showing that, although the wing is rescued (see Klein and Martinez Arias, 1998a), there is no induction of *wg* expression associated with the ectopic expression of *vg*.

and abolishes the development of the wing (Williams et al., 1994).

There are no mutations that remove the *vg*QE without affecting all functions of *vg* and which would allow us to test whether or not the growth of the wing requires the expression of *vg* through the *vg*QE. However, support for a requirement of the *vg*QE in the growth of the wing blade is provided by the clonal analysis, which demonstrates the requirement of *vg* activity for the survival of the cells in the wing pouch and by a recent report on the effects of dominant mutations in the *vg* gene (Kim et al., 1996; Simmonds et al., 1997). The dominant mutation vg^W leads to a very small wing pouch, which still contains a wing margin (Simmonds et al., 1997). Indeed detailed analysis of this mutation shows that, whereas vg^W mutant flies have *vg* function from the *vg*BE, the *vg*QE in completely inactive (Simmonds et al., 1997). Our results predict that the loss of the activity of the *vg*QE should lead to the loss of the wing pouch, but not of the margin, as is observed in the vg^w mutant. Interestingly, the presence of the vg^w mutation causes extensive cell death at the early third instar (Simmonds et al., 1997), at the time of extensive cell growth in the wing pouch. This cell death is probably due to the loss of *vg* expression in descendants of cells that are determined at the DV boundary and which come to lie outside the domain of the *vg*BE due to cell division. These cells would normally maintain the expression of *vg* (and therefore their fate) through the activation of the *vg*QE, which is inactive in the presence of vg^w. Several other recently reported results are in agreement with our conclusion: the clonal analysis suggests an autonomous requirement for *vg* by the cells of the wing pouch for survival (Simpson et al., 1981; Kim et al., 1996) and reduction of *wg* function results in the reduction of the size or complete loss of the pouch with *vg* expression reduced to the DV boundary (Couso et al., 1995; Zecca et al., 1996; Neumann and Cohen, 1996a,b).

Functional parameters in the development of the wing blade in *Drosophila*

Our results highlight some features of the process of wing development in *Drosophila*. In particular they highlight interactions between the *vg*BE and the *vg*QE which can explain how the wing grows and is patterned. During wing development, the *vg*BE has two well-defined roles. First, it is required to express *vg* in the primordium for the wing blade at the end of the second larval instar. Second, *vg*BE-mediated expression of *vg* in the primordium is a prerequisite to initiate the activity of the *vg*QE which will drive *vg* expression in the developing wing blade outside the domain of the *vg*BE. Thus, failures in the activity of the *vg*BE will result in failures in the activation of the *vg*QE and thus in the early stages of the development of the wing blade. Later expression of *vg* from the *vg*QE is likely to be sufficient to maintain the expression and function of *vestigial* (and identity) in the growing blade.

These interactions suggest a framework to consider how the wing blade develops and, in particular, about the interactions observed between the DV interface and the wing blade during the growth of the wing (Fig. 8). The primordium for the wing is defined, in the late second instar, precisely at the DV interface through interactions between Notch and Wingless signalling (Fig. 8; for further details see Klein and Martinez Arias, 1998a and Fig. 8A-C). These local activities are channelled through the *vg*BE and can account for the different mutant phenotypes and the regulatory and genetic interactions between these molecules.

In the early stages of the third instar, the primordium expands (Klein and Martinez Arias, 1998a,b), begins to grow and cells that come to lie outside the DV interface maintain the expression of *vg* through the activity of the *vg*QE (Fig. 8D). Clonal analysis and studies of cell proliferation (Milan et al., 1996) have not revealed any specific pattern to this phase of disc growth. However, it is clear that the DV interface and the AP boundary play some role in this process. Our results indicate that this role is likely to be the maintenance of the expression of *vg* through the *vg*QE (Fig. 8D,E) by the convergent effects of Vestigial, Wingless and Dpp. However, although *vg* as well as *wg* are required for the proper growth of the wing pouch, overexpression of these genes along the DV boundary does not lead to a larger pouch size (T. K. and A. M. A., unpublished data; see above), suggesting that the role of these genes during cell proliferation is more permissive rather than instructive.

At the beginning of the third instar, the wing primordium is subdivided into a proximal region, which will develop as hinge, and a distal one, which will develop as blade and upon which the margin will define the most distal point (Klein and Martinez Arias, 1998a, Klein et al., 1998). These subdivisions seem to occur in a sequence and as the wing blade grows they continue as revealed by the expression of *Dll* in a subset of cells within the wing blade. The expression of *Dll* requires Vestigial and is likely to define a domain within which high levels of Wingless signalling will induce bristle formation. Consistent with this, loss of *Dll* function results in the loss of bristles in the wing margin (Gorfinkiel et al., 1997). *Dll* delimits the distalmost region of the leg disc and its expression within the wing blade might reflect the homology that has been suggested to exist between the wing and the leg discs and highlights a clear proximodistal organization of the wing disc (Klein et al., 1998).

Analysis of the vg^1 and *Ser* mutant phenotypes suggests that the activity of *vg* in the initiation of wing development is only required for a limited period of time i.e. it is stabilized very quickly (Klein and Martinez Arias, 1998a,b). In both mutant situations although *vg* expression is initiated normally, it is rapidly lost and the mutant flies develop a small winglet, which is not observed in *ap* mutant flies or flies mutant for *vg* null alleles (T. K. and A. M. A., unpublished data). Therefore the short-lived activity of *vg* in these mutants is sufficient for the stable commitment of cells to the wing pouch fate. It is likely that *wg* plays a very important role in this commitment, which is reflected in its synergistic effects with *Notch*.

The contributions of the DV and AP axes to the patterning of the wing are becoming clear. However, little is still known about what triggers the foci of proliferation that drive the growth of the wing blade or how the interactions that we have described here control the final size of the wing. Our results here suggest a more indirect role of *wg* and *vg* in cell proliferation than previously suggested. Although the loss of function of each of these genes has a significant influence on the cell proliferation in the wing, the overexpression of both with the *vg*BEGal4 line do not lead to the increase of the wing pouch of the late third instar (see above). This suggests a more permissive role of the two genes in cell proliferation, probably

to maintain the identity of the wing pouch. Studies that suggest that the proliferation is induced by local clues and is a cell autonomous property (Milan et al., 1996) support this conclusion.

The role of Vestigial during the growth of the wing blade

vestigial encodes a nuclear protein with an unknown molecular function, but which can trigger the development of wing-tissue-specific properties upon developmentally unrelated groups of cells (Kim et al., 1996; Klein and Martinez Arias, 1998a and this work). Although there is no evidence that Vestigial interacts directly with DNA, our results suggest that it can influence patterns of gene expression and that this is probably the way in which it exerts its function during development.

It is possible that the role of Vestigial is to 'select' a combination of downstream genes that will lead to the development of wing tissue. In this situation, the interactions that we observe between Vestigial and the different signalling pathways might be essential in channelling the nuclear effectors of these signalling pathways to the promoters of genes that provide the identity of the wing. For example, *wingless* (Struhl and Basler, 1993; Wilder and Perrimon, 1995), *dpp* (Lecuit and Cohen, 1997) and *Notch* (Couso et al., 1994) are also required for the development and patterning of legs; however, if *vg* is expressed in cells from the leg discs, their development is altered and they develop wing tissue. This suggests that Vestigial provides an essential molecular context for wing development.

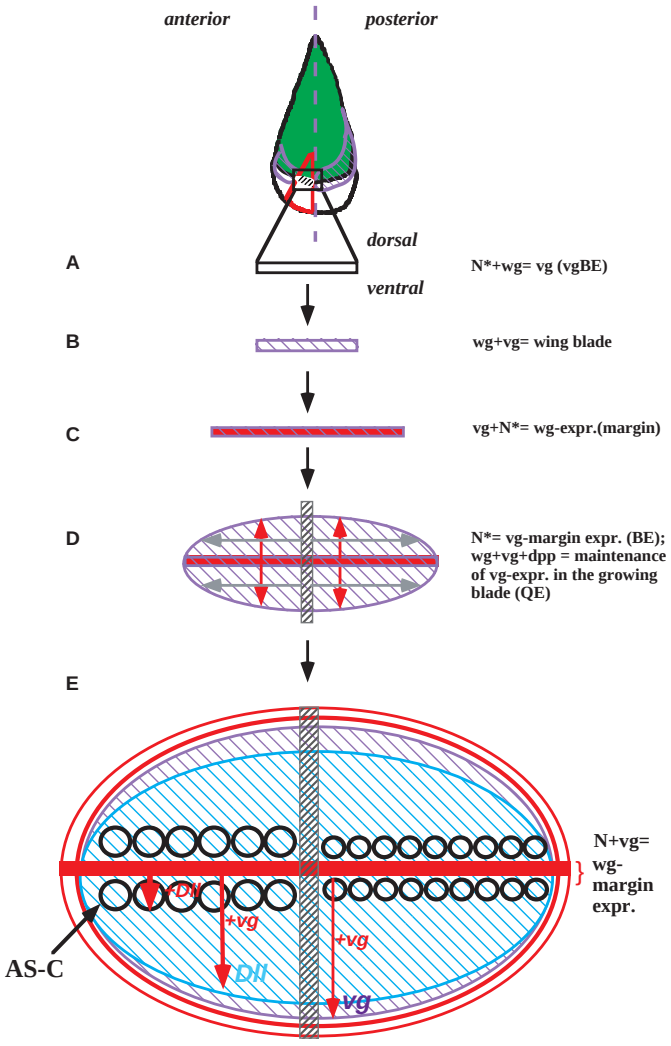

Fig. 8. Model for the sequential interactions that lead to the development and patterning of the wing blade (see text for further details). (A) In a first step, *Notch*/*Su(H)* signalling induces the expression of *vg* by activating the *vg*BE. The stabilization of this step requires the activity of *wg* and therefore only takes place where the activation of *Notch* intersects the activity of *wg*. This point also defines the future DV boundary and is highlighted by the square of the wing disc that is magnified in the cartoon. (B) Once *vg* is activated, it establishes the wing blade fate in collaboration with *wg* (see Klein and Martinez Arias, 1998a). (C) In a third step, *Notch*/*Su(H)* signalling and Vestigial collaborate to induce along the DV interface the expression of genes that are required for the establishment and patterning of the margin, among them *wg*. As a consequence of the restriction of *Notch*/*Su(H)* signalling to the DV interface, the initial primordium of the wing blade is, at this stage, a stripe that straddles the DV interface. (D) After the establishment of the expression of *wg* and *vg* in the wing primordium, the wing blade begins to grow. As cells proliferate, the expression of *vg* is maintained in the cells of the blade that lie outside of the domain of *Notch*/*Su(H)* signalling through the initiation and maintenance of the activity of the *vg*QE by Vestigial, Dpp and Wingless. The activity of the *vg*BE and the expression of *wg* at the wing margin continues to be controlled by *Notch*/*Su(H)* signalling and is therefore restricted to the DV boundary. The expression of *dpp* is restricted to a domain along the AP boundary through interactions between *hedgehog* and *patched*. Both Wingless and Dpp diffuse from their sources and, together with Vestigial, control *vg* expression in the wing blade through the *vg*QE. During the time of early wing development the expression of *wg* is changing rapidly and expands along the AP and the DV axes (see Klein and Martinez Arias, 1998a). This expansion is dependent on the *Notch* activity at the DV boundary (Klein and Martinez Arias, 1998b) and results in the recruitment of more cells into the wing primordium. A second consequence of this process is that cells at the periphery that are solely exposed to the activity of *wg* and therefore adopt the hinge fate (see Klein and Martinez Arias, 1998b for more details). (E) Diffusion from the DV boundary generates a gradient of Wingless that is translated in a gradient of expression of the Vestigial protein. Wingless and Vestigial collaborate in the developing wing blade to activate the expression of genes required for its further subdivision. In a first step the collaboration between Wingless and Vestigial results in the expression of Distalless within the domain of expression of *vg*. Loss of Dll function results in the loss of bristles and hairs at the wing margin (Gorfinkiel et al., 1997) in a manner similar to that caused by the loss of *wg* function. This suggests that, in the wild type, Dll is required together with high concentration of Wingless to induce the development of the bristles characteristic for the anterior margin (big circles) and the hairs characteristic for the posterior margin (small circles). This suggests a proximodistal organization of the wing that is generated in the course of development. Early on the wing anlage becomes subdivided along the proximodistal axis into the primordia for the proximal (hinge) and distal (blade). In the blade, the most distal point is defined as the wing margin (Couso et al., 1995; Klein et al., 1998). Later on the blade becomes subdivided into further domains reflected in the nested expression of Dll and members of the Achaete/Scute-Complex (AS-C).

Pattern formation is often described in terms of 'positional information'. In this paradigm, the informational input is thought to be universal, but its 'interpretation' is cell type specific (Wolpert, 1969). The widespread and recurrent use of a few signalling pathways in different developmental systems could be viewed as the proof of the universality of positional information, at least with regard to some of its elements. Our results suggest that Vestigial is an example of a key element in the interpretation of this information: wherever it is expressed, it channels the output of these signalling pathways into wing tissue. In some sense, Vestigial is an example of a molecule directly involved in the interpretation of positional information. It is likely that in different organs and structures, there are molecules that perform a similar role to that of Vestigial during wing development in *Drosophila*. Furthermore, the architecture of regulatory networks allows some flexibility as to the position and molecular nature of these molecular interpreters in the signalling networks.

Work during the last few years has begun to clarify the role of Vestigial during wing development (Williams et al., 1993, 1994; Kim et al., 1996, 1997a,b; Couso et al., 1995; Klein and Martinez Arias, 1998a). Analysis of epistatic relationships between genes involved in wing development has shown that vestigial does not lie at the bottom of a linear hierarchy (see e.g. Neumann and Cohen, 1996b; Zecca et al., 1996), but that it is a central element of a dynamic molecular network in which Wingless, Notch and Vestigial interact in a sequence of interdependent regulatory steps that set up the wing primordium development (Klein and Martinez Arias, 1998a,b and this work). The results presented here suggest that, during the growth of the wing, these interactions continue with the addition of one more element to the network, Dpp, and with Vestigial continuing to play a central role in the control of gene expression.

During the third larval instar, the expression of *vg*, like that of *Dll*, is regulated by Wingless (Kim et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1996b). However, we have shown here that Vestigial can itself regulate the expression of targets of Wingless, even in the absence of *wg* expression and that Vestigial, but not Wingless, can rescue the loss of wing development that results from the loss of *Notch* signalling (see above and Klein and Martinez Arias, 1998a). Since Vestigial itself is distributed in a gradient across the developing wing (Williams et al., 1991; Zecca et al., 1996; Neumann and Cohen, 1996b), these observations suggest that Vestigial might play a more instructive role in the development and patterning of the wing.

The results that we have presented here and elsewhere (see Klein and Martinez Arias, 1998a), raise questions about the proposal that Wingless acts as some sort of organizer during the development of the wing blade (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen 1996b, Zecca et al., 1996) but they also provide some insights as to what might be the actual function of Wingless. In the instances that we have tested, it seems that Wingless cannot elicit de novo gene expression alone and always acts synergistically with other signalling systems that direct gene expression during wing development. In particular, the function of *wg* appears to be to stabilize and maintain patterns of gene expression rather than direct patterns of gene expression. This function is very similar to the one that it plays in the embryo where Wingless does not initiate gene expression but rather reveals and maintains patterns of gene expression that have been established by other regulatory events (see Martinez Arias, 1998 for a review). Interestingly, a similar role for other Wnt proteins can be inferred from experiments that test their potential during the specification and patterning of the mesoderm in *Xenopus* (Cui et al., 1996; Hoppler and Moon, 1998).

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