Development 124, 21-32 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV8355

The *spalt* gene links the A/P compartment boundary to a linear adult structure in the *Drosophila* wing

Mark A. Sturtevant[1,2], Brian Biehs[1], Elizabeth Marin[1] and Ethan Bier[1,*]

[1]Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0116, USA
[2]Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011-5640, USA

*Author for correspondence

SUMMARY

During *Drosophila* embryogenesis, each segment is subdivided into an anterior and a posterior compartment through the action of the *engrailed* gene. Compartmental boundaries bisect imaginal disc primordia which give rise to adult appendages. In early larval development, a short-range Hedgehog signal originating from the posterior compartment of the imaginal wing disc activates expression of genes including *decapentaplegic* (*dpp*) in a stripe running along the anterior-posterior compartment boundary. Secreted Dpp emanating from the A/P boundary of wing discs then acts as a secondary signal to organize the wing over large distances. The transcription factor encoded by *spalt major* (*salm*) gene, which is expressed in a broad wedge centered over the *dpp* stripe, is one target of Dpp signaling. In this manuscript, we show that the anterior edge of the *salm* expression domain abuts a narrow stripe of *rhomboid* (*rho*)-expressing cells corresponding to the L2 longitudinal vein primordium. *hh* mis-expression along the anterior wing margin induces a surrounding domain of *salm* expression, the anterior edge of which abuts a displaced *rho* L2 stripe. *salm* plays a key role in defining the position of the L2 vein since loss of *salm* function in mosaic patches induces the formation of ectopic L2 branches, which comprise *salm*$^-$ cells running along clone borders where *salm*$^-$ cells confront *salm*$^+$ cells. These data suggest that *salm* determines the position of the L2 vein primordium by activating *rho* expression in neighboring cells through a locally non-autonomous mechanism. *rho* then functions to initiate and maintain vein differentiation. We discuss how these data provide the final link connecting the formation of a linear adult structure to the establishment of a boundary by the maternal Bicoid morphogen gradient in the blastoderm embryo.

Key words: wing vein, boundary, pattern formation, imaginal disc, *rhomboid, spalt, decapentaplegic*, *Drosophila*

INTRODUCTION

engrailed (*en*) expression in the posterior compartment of *Drosophila* imaginal discs activates transcription of the *hedgehog* (*hh*) gene which encodes a short-range diffusible signal (Tabata et al., 1992; Lee et al., 1992; Mohler and Vani, 1992; Zecca et al., 1995; Tabata et al., 1995). *en* also suppresses the response to Hh signaling (Sanicola et al., 1995; Zecca et al., 1995; Tabata et al., 1995). Because *en* activates *hh* expression while suppressing the response to Hh in the posterior compartment, the only cells that can respond to Hh lie along the anterior-posterior (A/P) border within the anterior compartment. This narrow strip of cells is within range of the Hh signal produced in the posterior compartment yet is not prevented from responding to Hh by *en* repression. A key gene activated by Hh in these cells is *decapentaplegic* (*dpp*) (Tabata and Kornberg, 1994; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Capdevila et al., 1994; Zecca et al., 1995; Ingham and Fietz, 1995; Tabata et al., 1995), which encodes a secreted protein (Dpp) in the TGF-β superfamily (Padgett et al., 1987). A variety of evidence suggests that Dpp then acts as a long-range signal to pattern and promote growth of imaginal discs (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Zecca et al., 1995; Ingham and Fietz, 1995; Nellen et al., 1996; Lecuit et al., 1996; Singer et al., 1997). One Dpp target gene is *salm*, which is expressed in a broad wedge centered over the *dpp* stripe in developing wing discs (see below and Nellen et al., 1996; Lecuit et al., 1996). Little is known, however, about the mechanisms by which signals emanating from the A/P boundary define the position of final differentiated adult structures.

Wing veins are one of the most conspicuous linear adult structures running perpendicular to the anterior-posterior axis. Genetic analysis of vein development has identified two clearly distinct stages in this process. During late larval development, the vein pattern is initiated independently on the primordia of both the dorsal and ventral surfaces of the wing as a series of sharp stripes (García-Bellido, 1977; Sturtevant et al., 1993). Subsequently, during pupal development, various forms of cell-cell signaling refine the vein pattern and consolidate the binary vein versus intervein cell fate decision (Waddington, 1940; García-Bellido, 1977; Díaz-Benjumea and García-Bellido, 1990; Sturtevant and Bier, 1995). Perhaps the most striking example of cell-cell communication during this latter

period is dorsal-to-ventral (D→V) induction, in which vein cells on the dorsal surface of the wing send a positive signal to underlying cells on the ventral surface to maintain their vein identity (García-Bellido, 1977; Sturtevant and Bier, 1995). The product of these various developmental events is the formation of a series of hollow tubular veins filled with fluid, which have components contributed by both the dorsal and ventral surfaces of the adult wing. Veins comprise densely packed living cells covered by a thick darkly pigmented cuticle and can be easily distinguished from intervein cells, which are tightly connected through the extracellular matrix to cells on the opposite wing surface and have a thin lightly pigmented cuticle with sparsely packed trichome hairs. Another difference between these two cell types is that vein cells survive into adulthood whereas intervein cells die upon eclosion.

One important gene required throughout wing vein development is *rhomboid* (*rho*). *rho* is expressed during the third larval instar in a series of stripes corresponding to longitudinal vein primordia and continues to be expressed in differentiating vein cells during pupal development (Sturtevant et al., 1993). *rho* is likely to function in veins by promoting signaling through the EGF-R pathway (Sturtevant et al., 1993). In contrast to *rho*, other known components of the EGF-R signaling pathway are expressed ubiquitously during vein development (Sturtevant et al., 1994, M. A. Sturtevant and E. Bier, unpublished results). The localized pattern of *rho* expression is required for generating the normal vein pattern since lack of *rho* function leads to vein loss phenotypes while mis-expression of *rho* induces formation of ectopic veins (Sturtevant et al., 1993; Noll et al., 1994).

In the current study, we link early anterior-posterior signaling from the A/P compartment boundary to the formation of the second longitudinal vein (L2). We also extend previous observations (Sturtevant and Bier, 1995) suggesting that the wing disc is subdivided into a series of discrete sectors bounded by longitudinal vein primordia. We show that the L2 vein primordium forms at the edge of a broad domain expressing the *spalt major* (*salm*) gene. *rho*-expressing L2 precursor cells do not express *salm* but are adjacent to cells that do. In addition, we show that mis-expression of *hh* along the anterior wing margin, which indirectly activates *salm* expression at a distance, displaces the position of the L2 vein to the edge of the new $salm^+$ territory in imaginal discs and pupal wings. Finally, we show that a +/– *salm* boundary is sufficient to induce formation of an ectopic L2 vein which lies within and courses along the edge of the $salm^-$ clone where it confronts $salm^+$ cells. Furthermore, ectopic islands of triple row bristles, typical of the junction between L2 and the marginal vein, are generated where $salm^-$ clones extend to the margin. These results suggest that the L2 vein is induced at the boundary between $salm^-$ and $salm^+$ cells.

The results in this paper are consistent with data presented in the accompanying paper (Singer et al., 1997) in which similarly branched L2 veins are observed in clones lacking function of the type I Dpp receptor chains encoded by the *saxophone* (*sax*) and *thick veins* (*tkv*) genes. As such *sax* and *tkv* clones reduce or eliminate the expression of *salm* (Singer et al., 1997), it is likely that a specific threshold response to Dpp determines the position of the L2 vein. We discuss how these data provide the final link in an uninterrupted chain of genetic events connecting the formation of a linear adult structure (L2) to a boundary generated in the blastoderm stage embryo by the Bicoid morphogen gradient. We propose that other longitudinal veins may form by analogous mechanisms at borders between discrete subdivisions of the wing imaginal disc.

MATERIALS AND METHODS

Fly stocks

All genetic markers and chromosome balancers used are described in Lindsley and Grell (1968) and Lindsley and Zimm (1992). We thank Joan Hooper (University of Colorado Health Science Center, Denver) for the hh^{Mrt} stock, Dr Walter Gehring (Biozentrum, University of Basel, Basel, Switzerland) for the A405.1M2 *salm-lacZ* enhancer trap stock, Dr Elizabeth Knust (Universität zu Köln, Köln, Germany) for the *ptc*-GAL4 stock, Dr Andrea Brand (Welcome Institute, Cambridge, UK) for the GAL4-69B stock, Dr Reinhard Schuh (Max-Planck-Institut, Göttingen, Germany) for the $salm^{16}$ allele, and Dr Gary Struhl (Columbia University College of Physicians, New York) for stocks carrying a HS-FLP source and a *dpp* flip-out cassette. Other stocks were obtained from the Bloomington, Indiana and Bowling Green, Ohio *Drosophila* Stock Centers.

Mosaic analysis

Clones were generated using the FLP-FRT recombinase system of Golic (Golic, 1991). Larvae of the genotype HS-*Flp*; *ck* $salm^{IIA}$ FRT^{40A}/FRT^{40A} or HS-*Flp*; $salm^{16}$ FRT^{40A}/FRT^{40A} were heat shocked during the first and second larval instars to generate mosaics. As approximately one third of adults of these genotypes had significant defects in vein patterning near L2 (see Fig. 3), homozygous $salm^-$ clones were generated at high frequency. Clone boundaries were scored by the recessive *ck* trichome marker under a compound microscope. *dpp* flip-out experiments were performed by crossing flies carrying the (tub>*forked*>*dpp*) flip-out cassette to flies carrying a HS-FLP insertion and then heat shocking according to Zecca et al. (1995).

Mounting fly wings

Wings from adult flies were dissected in isopropanol and mounted in Canadian Balsam mounting medium (Gary's magic mountant) following the protocol of Lawrence et al. (in Roberts, 1986).

In situ hybridization to whole-mount embryos or discs

In situ hybridization using digoxigenin-labeled antisense RNA probes (O'Neill and Bier, 1994) was performed alone or in combination with anti-β-galactosidase labeling as described in Sturtevant et al. (1993).

RESULTS

Longitudinal vein formation is initiated at boundaries between discrete sectors of the wing imaginal disc

One reason for suspecting that a series of sharp boundaries partitions the mid-third instar wing imaginal disc is that *rho* expression is initiated in a pattern of narrow stripes running perpendicular to the future wing margin at this time (Fig. 1A). These stripes of *rho* expression correspond to longitudinal vein primordia (Sturtevant et al. 1993) in which *rho* functions to promote vein formation by locally enhancing EGF-R activity (Sturtevant et al. 1993; Noll et al., 1995; Sturtevant and Bier, 1995). *rho* expression becomes stronger during the late third instar (Fig. 1B) and is maintained in vein primordia throughout pupal development (and Fig. 1E).

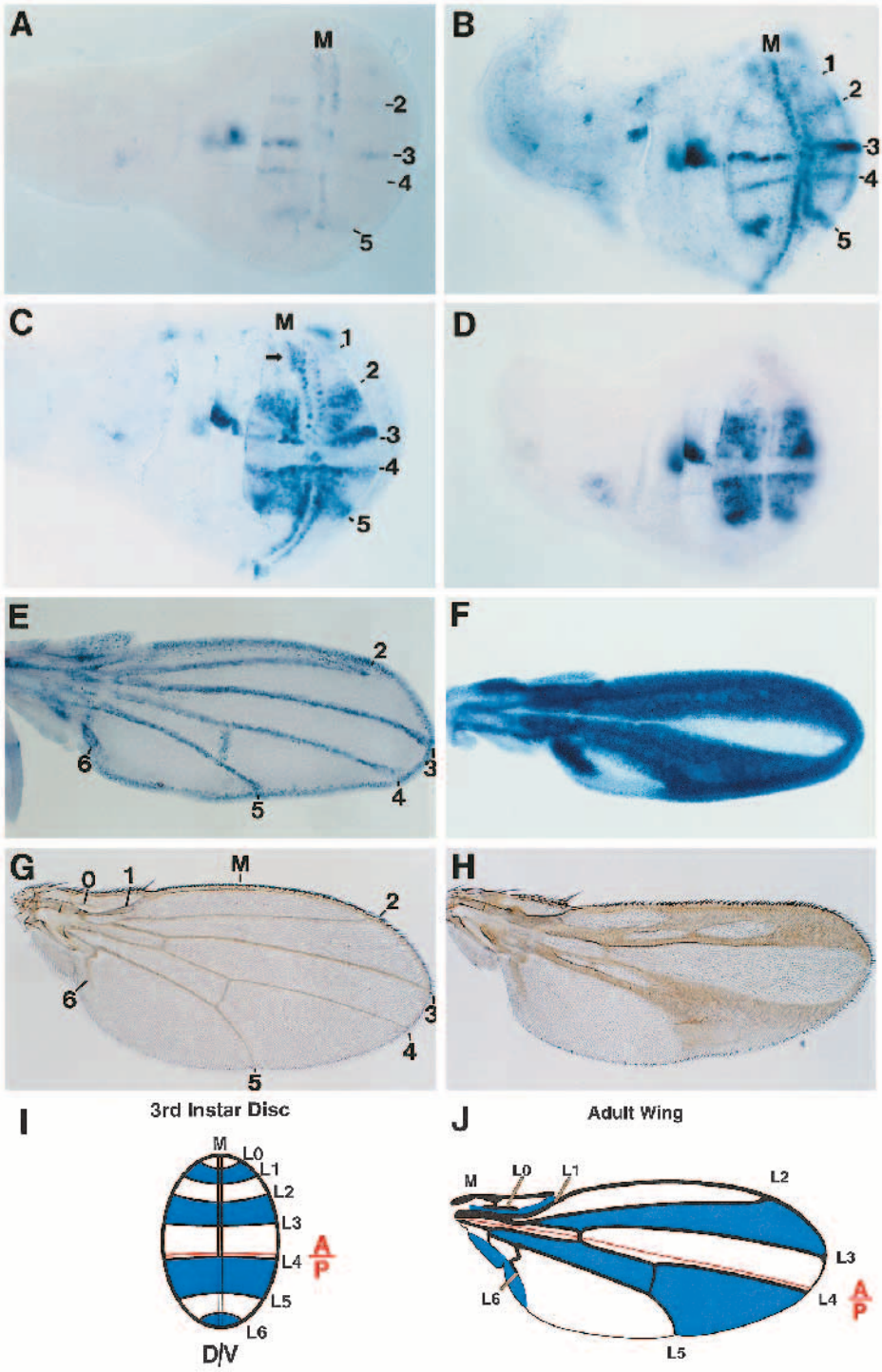

Fig. 1. Vein primordia form at boundaries between discrete sectors of the wing imaginal disc. (A) In situ hybridization of a wild-type mid-third larval instar imaginal wing disc with a digoxigenin-labeled antisense *rho* RNA probe. *rho* is initiated in a series of sharp stripes corresponding to longitudinal vein primordia. The marginal vein (M) and L2-L5 longitudinal vein primordia are labeled 2-5. L0, L1 and L6 are difficult to identify at this stage and may not be specified until later developmental stages. (B) *rho* expression in wild-type late third larval instar imaginal wing disc. Veins are labeled as above. L1 can be identified by this stage. (C) *rho* expression in a *net/net* late third larval instar wing disc. The arrow indicates the location of a small anterior ectopic domain of *rho* expression. (D) *rho* expression in a *Notch*/+; *net/net* mid-third larval instar wing disc. Solid blocks of ectopic *rho* expression alternate with regions devoid of *rho* expression. (E) *rho* expression in a wild-type pupal wing. Expression is confined to developing veins. Longitudinal veins L2-L6 are labeled. L0 and L1 can also be clearly resolved at this stage. (F) *rho* expression in a $Notch^{ts}/Notch^{ts}$; *net/net* pupal wing isolated from an individual shifted from the permissive temperature (18°C) to the non-permissive temperature (29°C) at the beginning of pupariation. Sectors of solid vein (*rho* expression) are separated by non-expressing intervein regions. Although such temperature shifted individuals do not eclose as adults, pharate adults can be isolated which have solid sectors of vein alternating with purely intervein regions (M. A. Sturtevant and E. Bier, unpublished results). (G) A wild-type adult wing. Longitudinal veins L0-L6 are labeled 0-6. The margin, which represents the most anterior vein, is labeled M. (H) A N^{55e11}/+; *net/net* adult wing. (I) Diagrammatic representation of potential subdivisions of the third instar disc into sectors bounded by vein primordia (L0-L6). Regions shaded in dark blue include those corresponding to the areas of observed ectopic *rho* expression in *net* mutants. The L4 primordium initially abuts the A/P boundary, which is labeled in red, but then rapidly becomes separated from it by one cell diameter. (J) Diagrammatic representation of how the potential subdivisions of the third instar disc shown in panel I would map onto the final adult wing.

Another line of evidence supporting the existence of sharp borders between discrete domains of the wing disc is the abnormal pattern of *rho* expression in *net* mutant discs (Sturtevant and Bier, 1995), which consists of broad domains of cells ectopically expressing *rho* alternating with regions devoid of *rho* expression (Fig. 1C). An important feature of *rho* mis-expression in *net* mutant discs is that wild-type expression of *rho* in vein primordia, which is slightly stronger than ectopic *rho* expression, is observed at the boundaries between these domains (Fig. 1C). The juxtaposition of ectopic *rho*-expressing sectors with regions devoid of *rho* expression is yet more pronounced in *Notch* (*N*); *net* double mutant discs (Fig. 1D). This pattern of ectopic *rho* expression persists in *N*; *net* mutant pupae (Fig. 1F, compare to Fig. 1E)

and is ultimately reflected in the phenotype of *N*; *net* mutant adult wings, which are composed of bands of solid vein cuticle alternating with sectors having purely intervein morphology (Díaz-Benjumea and García-Bellido, 1990; Fig. 1H; compare with Fig. 1G). These data are illustrated schematically in Fig. 1I, in which vein primordia form at the boundaries between distinct territories of cells. These alternating sectors of the wing disc map to corresponding domains of the adult wing delimited by longitudinal veins (Fig. 1J).

The L2 vein primordium directly abuts the anterior edge of the *salm* expression domain

A candidate gene for defining the location of a longitudinal vein primordium is *salm*. *salm* encodes a putative transcription factor, which functions in concert with homeotic genes to establish cell fates during embryogenesis (Kühnlein et al., 1994). *salm* is expressed in a broad central domain approximately centered over the middle of the wing disc (Fig. 2A). We performed double-label experiments on *salm-lacZ* wing imaginal discs to visualize the pattern of *dpp* RNA relative to *salm*-β-galactosidase (β-gal). In second instar discs, *salm-lacZ* expression straddles the narrower *dpp* stripe extending 2-3 cells beyond the 6- to 8-cell-wide *dpp* domain (data not shown). During the third larval instar, following significant cell proliferation, the edges of the *salm* expression domain sharpen. The distance between the anterior edge of the *salm-lacZ* expression domain and the *dpp* stripe, which remains a constant 6-8 cells wide, increases to approximately 12 cells (data not shown, see Nellen et al., 1996; Lecuit et al., 1996).

Double-label experiments with *rho* and a *salm-lacZ* enhancer trap line reveal that the sharp anterior edge of the broad *salm* expression domain directly abuts the L2 vein primordium in third instar wing discs (Fig. 2A). The posterior edge of *salm* expression runs equidistantly between L4 and L5 and therefore does not coincide with a vein boundary in *Drosophila*. However, we believe that this border does correspond to a vein forming boundary in more primitive insects, which have twice as many wing veins as *Drosophila* (M. A. S. and E. B., unpublished data).

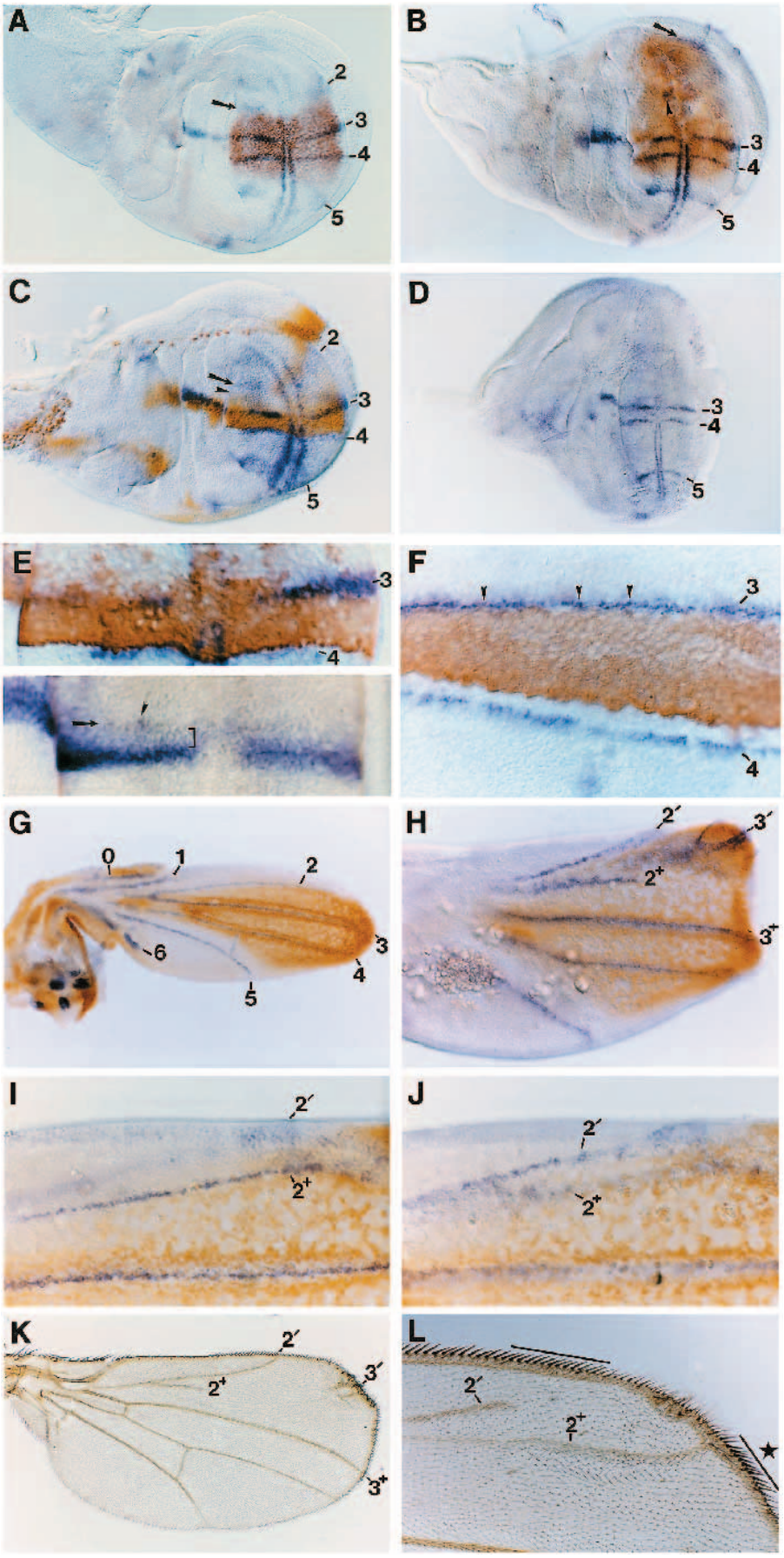

Fig. 2. The L2 vein forms immediately anterior to the *salm* expression domain. Wing veins are indicated as in Fig. 1. (A) The pattern of *salm-lacZ* expression relative to vein primordia as revealed by β-galactosidase (β-gal) expression driven from the A405.1M2 enhancer trap insertion (Wagner-Berholz, 1991) in a late third instar imaginal wing disc. β-gal protein was detected with an anti-β-gal antibody (visualized by a brown peroxidase reaction product) and vein primordia were visualized by in situ hybridization to a *rho* probe (visualized by a blue alkaline phosphatase reaction product). The anterior boundary of the *salm-lacZ* staining territory abuts L2 (arrow). The posterior edge of the *salm-lacZ* domain runs midway between the L4 and L5 primordia. (B) *salm-lacZ* expression in a late third instar hh^{Mrt} imaginal wing disc. *rho* is expressed in an arc following the outer edge of the *salm* expression domain (arrow). In hh^{Mrt} discs such as the one shown, which have a large domain of ectopic *salm* expression, a vein likely to have L3 identity (i.e. which gives rise to a dorsal vein marked with campaniform sensilla - see panel K) is often induced close to the margin. Although this presumed L3 expression is difficult to see in this disc (arrowhead), it is very pronounced in other discs with similar degrees of ectopic *salm* expression. (C) The pattern of *dpp* expression relative to vein primordia revealed by *lacZ* expression driven from a *dpp-lacZ* enhancer trap in a late third instar imaginal wing disc. β-gal protein was detected with an antibody (brown peroxidase reaction product) and vein primordia were visualized by *in situ* hybridization to a *rho* probe (blue alkaline phosphatase reaction product). There are two distinct domains of *dpp-lacZ* expression. One domain, in which there is strong β-gal labeling near the L3 and L4 primordia, corresponds closely to cells expressing *dpp* RNA at this stage. A second weaker domain of β-gal expression extends beyond the strong domain in the anterior direction. The anterior border of this latter domain (arrowhead) lies closer to the L2 primordium (arrow) than to L3. (D) The pattern of vein primordia is altered by ectopic expression of *dpp*. The wing disc shown here was derived from an individual of the genotype HS-FLP; *dpp* flip-out in which the HS-FLP was induced during the early second larval instar. This treatment frequently resulted in the formation of elongated wing pouches with posteriorly displaced L5 veins and anteriorly shifted L2 veins (not visible on this disc). In contrast, the spacing of the L3 and L4 vein primordia is relatively normal. (E) The *ptc* expression domain as revealed in *ptc-GAL4*; *UAS-lacZ* discs (Speicher et al., 1994), is bounded by the L3 and L4 *rho*-expressing vein primordia (upper panel). *ptc* RNA expression, however, is graded being strongest near L4 and fading to the low level typical of the anterior compartment near L3 (lower panel). The bracket (lower panel) indicates the zone of moderate *ptc* expression. The anterior limit of this zone of intermediate *ptc* expression corresponds approximately to the line of Rho-expressing L3 cells (arrow). The arrowhead points to a punctate plaque of Rho staining. (F) The β-gal expression domain in *ptc-GAL4*; *UAS-lacZ* pupal wings is largely bounded by the L3 primordium in the anterior direction (although a minority of L3 cells also express β-gal, arrowheads) and by a line likely to be the A/P boundary in the posterior direction, which is separated from L4 by a narrow strip of unlabeled cells. *ptc* RNA is expressed at elevated levels in a similar central wedge during early pupal stages (data not shown). (G) A wild-type wing double labeled with an anti-β-gal antibody to visualize expression from the *salm-lacZ* enhancer trap and with an antisense *rho* RNA probe to label veins. The limits of the central domain of *salm* expression are in the same positions relative to longitudinal veins as in third instar discs. Thus, the anterior edge of the *salm* domain directly abuts L2 and the posterior border runs between L4 and L5. In addition to the central *salm* domain, there are two other domains of *salm* expression in pupal wings. One of these lies between L0 and the anterior margin and the other is sandwiched between L6 and the posterior margin. Longitudinal veins L0-L6 are indicated (0-6). (H) *salm-lacZ* expression (brown) relative to *rho* expression (blue) on the ventral surface of a hh^{Mrt} pupal wing. This vein bifurcates with the anterior branch (2′) following the displaced *salm* contour and the posterior branch (2^+) forming in the location of the normal L2 vein. The basis for this forked L2 phenotype is the same as that described for a wing shown at higher magnification in panels I and J. Note also the ring of ectopic *rho* expression within the *salm* domain near the margin which most likely represents an ectopic L3 vein (labeled 3′). The normal L3 vein (3^+) is also indicated. (I) *salm-lacZ* expression (brown) relative to *rho* expression (blue) on the dorsal surface of a hh^{Mrt} pupal wing is virtually indistinguishable from wild-type. Note that *rho* expression in the dorsal component of L2 (2^+) follows the perimeter of the *salm* expression domain. The anteriorly shifted L2 vein on the ventral surface appears as a faint blur since it is largely out of focus in this panel except at the very distal tip (marked 2′). (J) *salm-lacZ* expression (brown) relative to *rho* expression (blue) on the ventral surface of the same hh^{Mrt} pupal wing shown in panel I. The ventral component of the L2 vein (2′) follows the anteriorly expanded *salm* expression domain. The posterior fork in L2 (2^+), which extends into the *salm* expression domain, is presumably the result of the dorsal component (panel I) inducing a ventral component in the usual location of L2 by a D→V inductive signal. Because the primary dorsal and ventral vein components are not aligned, a forked L2 vein is generated. (K) A hh^{Mrt} adult wing. The anterior branch (2′) of the forked L2 vein is greatly displaced (in the anterior direction) relative to wild-type. The posterior branch (2^+), however, is in the normal location of an L2 vein. There is also a patch of ectopic vein material near the margin (3′) which is likely to have L3 identity since it is always a dorsal vein and is frequently decorated with campaniform sensilla, which are normally only observed on L3 veins. (L) A high magnification view of the margin of a hh^{Mrt} wing. Note the island of triple row bristles (overlined and labeled by a star) where the posterior branch of L2 (2^+) intersects the margin at the normal L2 position which is separated from the continuous (anteriorly shifted) row of triple bristles (overlined only).

Other longitudinal veins abut gene expression boundaries

We also determined the position of *rho*-expressing vein primordia relative to the overlapping *dpp* and *patched* (*ptc*) stripes in *dpp-lacZ* discs (Fig. 2C) and in *ptc-GAL4*; *UAS-lacZ* discs (Fig. 2E, upper panel) and pupal wings (Fig. 2F). We observed that the central stripes of *dpp-lacZ* and *ptc-GAL4-*; *UAS-lacZ* expression are bounded on the posterior edge by the L4 primordium. Double-label experiments with *hh-lacZ* and *rho* also confirm that *rho* expression in the L4 primordium initially abuts the compartment boundary (data not shown). In slightly older discs, a small gap forms between the posterior border of the *dpp-lacZ* or *ptc-GAL4*; *UAS-lacZ* domains and the L4 *rho* stripe (data not shown). As the posterior edge of *dpp* expression abuts the A/P compartment boundary (Posakony et al., 1991; Tabata and Kornberg, 1994), these data indicate that the L4 stripe initially abuts the compartment border and that, subsequently, cells in immediate contact with the boundary cease expressing *rho*. This leads to displacement of the A/P border from the L4 primordium (Fig. 2F).

In the anterior direction, strong *dpp-lacZ* expression extends one or two cell diameters beyond the L3 primordium (Fig. 2C). In addition to the domain of intense β-gal staining, there is a domain of lower expression extending more than half way between L3 and L2 (arrowhead). This domain of weak β-gal expression may reflect β-gal protein perdurance in cells that

expressed *dpp* during earlier stages of larval development. Interpretation of this low level *dpp-lacZ* expression as β-gal perdurance is consistent with the observation that the *dpp* stripe, which remains a constant 6-8 cells across, comprises a greater fraction of the width of second instar discs than of third instar discs (see above). Lecuit et al. (1996) propose a similar temporal explanation for activation of the *omb* gene in a broader stripe than *salm* by an activated Tkv* receptor expressed under the control of the dpp^{disk} promoter.

In *ptc-GAL4*; *UAS-lacZ* discs, the anterior edge of the β-gal stripe, which is narrower than that observed in *dpp-lacZ* discs, runs along the L3 primordium. *ptc-GAL4*; *UAS-lacZ* expression abuts, but is largely excluded from, the L3 *rho* stripe in larvae (Fig. 2E, upper panel) and pupae (Fig. 2F), although a minority of L3 cells do label for β-gal (e.g. arrows in Fig. 2F). The stripe of endogenous *ptc* RNA expression comprises a strong expressing domain and a zone of less intense expression (Fig. 2E, lower panel). The anterior border of the zone of weaker *ptc* expression appears to be in the vicinity of Rho-expressing L3 cells (Fig. 2E, lower panel). The difference observed between the levels of endogenous *ptc* RNA versus *pct-GAL4*; *UAS-lacZ* driven β-gal protein presumably reflects stability of the β-gal protein and strong expression driven by the GAL4-UAS system.

The L2 primordium abuts the anterior edge of the *salm* domain in hh^{Mrt} mutant wings

We have also examined the pattern of vein primordia with respect to positional markers in mutant wing discs. The spacing of the L2 and L5 *rho* stripes is sensitive to the level of *dpp* since various forms of *dpp* mis-expression generate discs with an elongated A/P axis and with L2 and L5 *rho* stripes shifted away from the A/P border (Fig. 2D). In contrast, the distance between the L3 and L4 *rho* stripes appears to be unaffected in such discs. These data suggest that the positions of the L2 and L5 vein primordia may be determined by threshold responses to Dpp produced at the A/P boundary.

To test whether the position of the L2 primordium might be linked to the anterior border of *salm*, we examined expression of *rho* and *salm-lacZ* in hh^{Mrt} mutant wings. In hh^{Mrt} wing discs, mis-expression of *hh* along the anterior wing margin induces *dpp* expression along the margin, which in turn activates ectopic *salm* expression at a distance (data not shown; see Nellen et al., 1996 and Lecuit et al., 1996 for a detailed

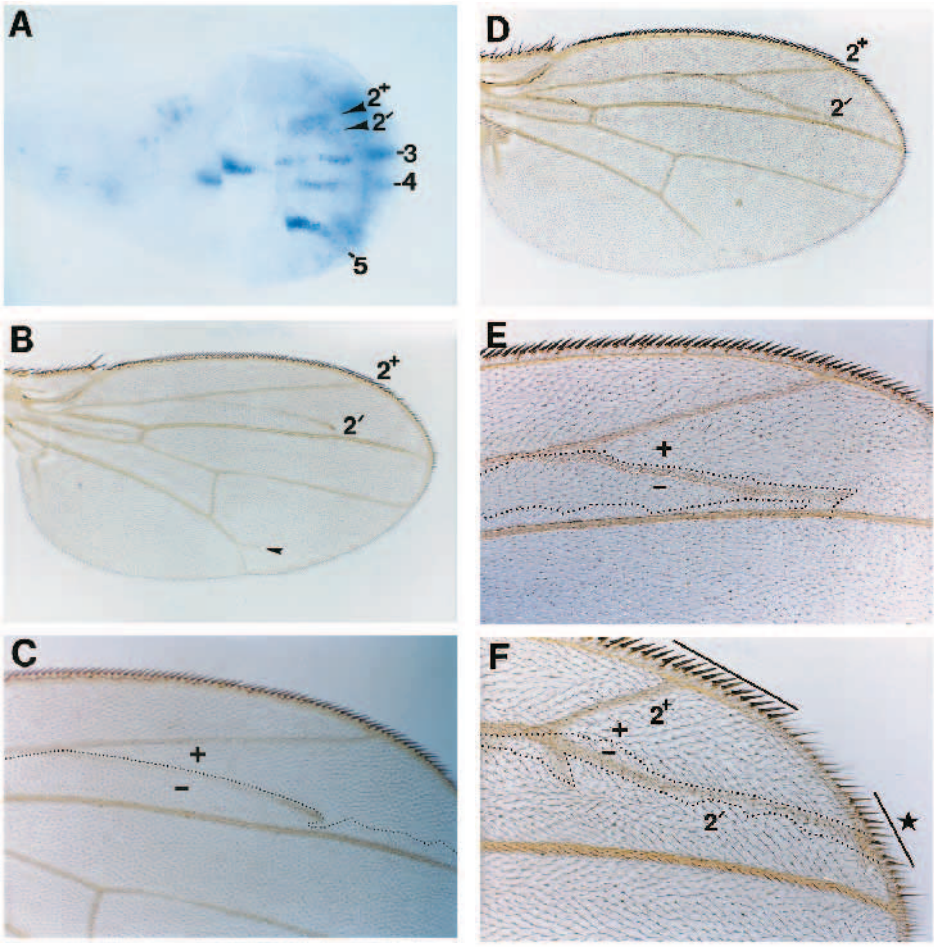

Fig. 3. *salm* is required for establishing the L2 boundary. (A) *rho* expression in a *salm* mosaic disc in which L2 is branched on the presumptive dorsal surface. The arrowhead labeled 2^+ indicates the normal L2 primordium and the arrowhead labeled 2′ indicates the novel L2 branch in a disc presumed to have a $salm^-$ clone in this region. The primordia of L3-L5 are also indicated. (B) An adult wing containing a dorsal *ck* $salm^{IIA}$ mosaic clone and a long ectopic L2 fork (labeled 2′) which branches in the posterior distal direction from the normally positioned L2 vein (labeled 2^+). The forked L2 phenotype results from loss of *salm* function since identical phenotypes were observed when clones were induced using either the $salm^{IIA}$ or the $salm^{16}$ (null) alleles. The arrowhead indicates an ectopic vein segment branching from the L5 primordium. (C) A higher magnification of the wing presented in panel B showing that the ectopic L2 vein forms within and at the extreme anterior edge of the marked *ck* $salm^{IIA}$ clone. The dotted line shows the anterior limit of the large $salm^-$ clone. The – symbol indicates mutant cells of the genotype *ck salm* /*ck salm* and + indicates wild-type cells which are either of the genotype *ck salm*-/+ or +/+. (D) An adult wing containing a ventral *ck* $salm^{IIA}$ mosaic clone and a long ectopic posterior branch (2′). The wild-type L2 vein (2^+) is also indicated. (E) A higher magnification of the wing presented in panel D showing that the ectopic vein forms within and along the border of the marked *ck* $salm^{IIA}$ clone. (F) A high magnification view of a wing margin in which a *ck* $salm^{IIA}$ clone extends to the margin and induces an island of ectopic triple row bristles (overlined and labeled with a star) separated from the normal triple row of bristles (overlined) by an intervening region of double row bristles. This ectopic island of triple row bristles may be partly non-autonomously induced as the formation of triple row bristles in both the anterior and posterior directions extends beyond the limits of the *ck* clone. However, since the *ck* marker cannot be scored along the margin itself, it is possible that the clone does extend up and down along the margin.

demonstration of the dependence of *salm* expression on the level of Dpp activity). Ectopic expression of *salm* in hh^{Mrt} mutant wings can be observed in late second instar discs (data not shown), revealing that the pattern of *salm* expression precedes that of *rho* expression in vein primordia by more than 16 hours in both wild-type (see above) and hh^{Mrt} mutants. As in wild-type third instar discs, a stripe of *rho* expression follows the contour of ectopic *salm* expression (Fig. 2B, arrow). We also observed ectopic *rho* expression running near and parallel to the margin within the ectopic *salm* expression domain (Fig. 2B; arrowhead). This latter expression of *rho* inside the ectopic *salm* domain most likely represents induction of L3 vein fates since these cells lie within the narrow domain of *dpp* expression as does the normal L3 vein (see below and legends to Fig. 2H,K). In situations where smaller ectopic domains of *salm* expression are observed, expression of *rho* internal to the ectopic *salm* domain is generally not observed (data not shown). This suggests that high levels of the Hh signal are required to induce *rho* expression in L3 and that lower levels are sufficient to trigger expression of *rho* in L2. In the most severely affected hh^{Mrt} wing discs, we observed that the wild-type and ectopic *salm* expression domains were fused into a single large domain (e.g. Fig. 2B). In such cases, *rho* expression in the position of the normal L2 primordium was lost.

rho and *salm* continue to be expressed in abutting patterns during pupal stages (Fig. 2G) when various forms of cell-cell communication refine the vein pattern. As during earlier developmental stages, the *rho* L2 stripe lies immediately anterior to the broad central domain of *salm* expression. In addition, there

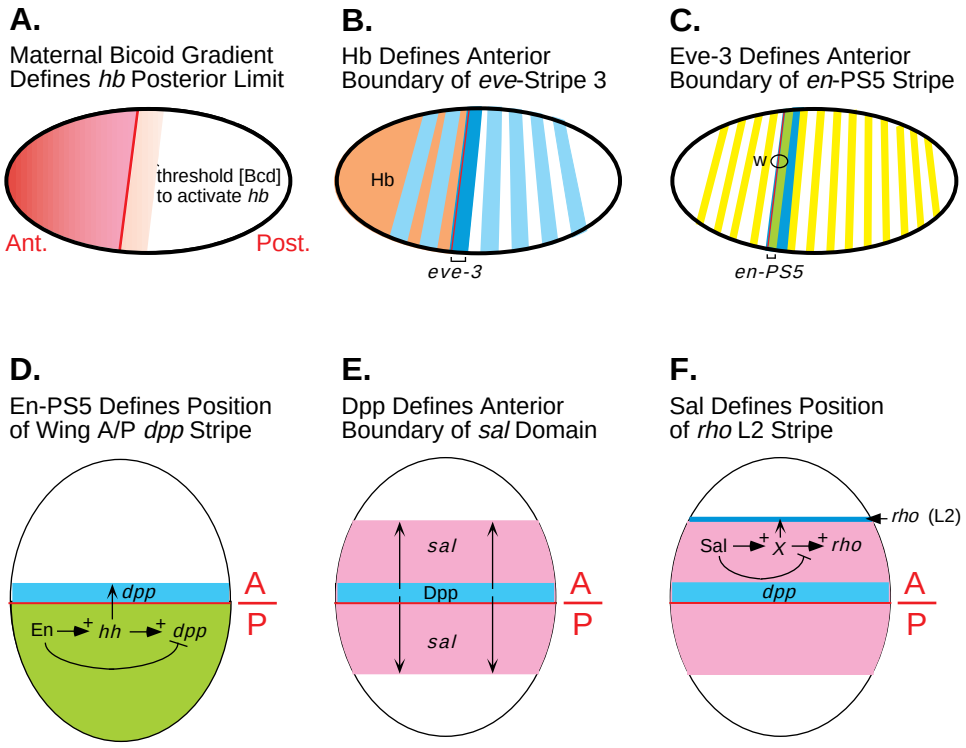

Fig. 4. A sequence of localized gene action during embryonic and imaginal disc development specifies the position of the L2 vein. (A-C) Embryos are depicted with anterior facing to the right and dorsal at the top. (D-F) Wing imaginal discs are depicted with the axes rotated 90° relative to the embryos shown in A-C for graphic clarity. In D-F, anterior is at the top and posterior at the bottom. (A) The Bicoid morphogen gradient activates zygotic *hunchback* (*hb*) expression in the early precellular blastoderm embryo (Shroeder et al., 1988; Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989; Simpson-Brose et al., 1994). The sharp decline in *hb* expression results from a threshold response (red line) to Bicoid activation. (B) The posterior edge of Hb sets the anterior limit of *even-skipped stripe 3* (*eve-3*) by repressing *eve-3* expression in more anterior cells (Small et al., 1996). In hb^{-} mutants, this boundary moves forward. (C) During the late blastoderm stage of embryogenesis, *engrailed* (*en*) expression is initiated in the posterior compartment of each segment (yellow stripes). Activation of *en* expression in parasegment 5 (PS5) is dependent on *eve-3* (green stripe = yellow *en-PS5* stripe superimposed on blue *eve-3* stripe) (DiNardo and O'Farrell, 1987; Lawrence et al., 1987; Frasch et al., 1988). Thus, the anterior edge of *en-PS5* is determined by the anterior boundary of *eve-3*. The anlagen for the wing imaginal disc (W) is bisected by the anterior border of *en-PS5*. *en* expression in posterior compartment cells is maintained and refined by a series of mechanisms during gastrulation (Heemskerk et al., 1991; see Perrimon, 1995 for review). *en* expression in posterior compartment cells (Kornberg et al., 1985) prevents these cells from mixing with anterior compartment cells (Morata and Lawrence, 1975; Lawrence and Morata, 1976; Lawrence and Struhl, 1982). (D) A magnified view of the wing imaginal disc showing that one gene activated by *en* is *hedgehog* (*hh*) (Tabata et al., 1992, 1995; Sanicola et al., 1995; Guillen et al., 1995; Zecca et al., 1995), which encodes a short-range signal (Hh). During early larval development, Hh signaling activates *dpp* expression in a narrow stripe of anterior compartment cells abutting the posterior compartment (Basler and Struhl, 1994; Capdevila et al., 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994). *en* also functions to prevent *dpp* expression in the posterior compartment (Sanicola et al., 1995; Zecca et al., 1995; Tabata et al., 1995). (E) Dpp diffusing from its site of production along the A/P compartment boundary determines the limits of *salm* expression in a threshold-dependent manner (Nellen et al., 1996; Lecuit et al., 1996; Singer et al., 1997; data presented here). (F) The anterior boundary of the broad *salm* expression domain defines the position of *rho* expression in the L2 vein primordium and *rho* then initiates vein development (Sturtevant et al., 1993; Sturtevant and Bier, 1995). Since *salm* encodes a transcription factor, it is likely that this non-autonomous action of *salm* is mediated by an extracellular signal (X). Because L2 *rho* expression is excluded from *salm*-expressing cells, we propose that *salm* may also function autonomously to repress *rho* expression in the vicinity of L2. Presumably *rho* expression in other veins such as L3 and L4 is mediated by entirely different mechanism(s), which are not subject to repression by *salm*. The link between the anterior edge of *salm* expression and the position of the L2 primordium makes it possible to trace the origins of a linear adult structure (i.e. L2) to a boundary defined during the blastoderm stage of embryogenesis by the maternally generated Bicoid morphogen gradient (i.e. the posterior limit of zygotic *hb* expression) through an uninterrupted chain of localized gene action.

are two domains of *salm* expression between L0 and the anterior margin and between L6 and the posterior margin which can be resolved during this period (Fig. 2G).

rho L2 stripes also course along the outside perimeter of ectopic *salm*-expressing domains in hh^{Mrt}/+ pupal wings (Fig. 2H,J). Due to the highly variable expressivity of the hh^{Mrt}/+ allele, ectopic domains of *salm* expression frequently form asymmetrically with respect to the wing margin and may lie predominantly on either the dorsal or the ventral surface of the wing. When ectopic expression of *salm* is confined to one surface of the wing, the dorsal and ventral components of the L2 vein are driven out of register. This divergence of the dorsal and ventral vein components leads to the formation of forked L2 veins (Fig. 2H,J), most likely as a result of D→V induction (García-Bellido, 1977). For example, in the hh^{Mrt}/+ pupal wing shown in Fig. 2I,J, ectopic *salm* expression is restricted to the ventral surface of the wing (Fig. 2J). The anterior branch of the L2 vein on the ventral wing surface (2′) follows the anteriorly shifted *salm* contour on the ventral surface (Fig. 2J). The posterior branch of this same vein (2^+) is observed in the position of the wild-type L2 primordium and underlies the L2 vein component on the nearly wild-type dorsal surface of the wing (labeled 2^+ in Fig. 2I). Thus, the posterior fork of the ventral L2 vein is likely to have been induced by D→V signal(s) emanating from the unaltered dorsal component of L2. Similar forked L2 vein phenotypes can result from dorsally restricted ectopic *salm* expression (data not shown). These pupal vein phenotypes prefigure the final bifurcated L2 vein pattern observed in hh^{Mrt}/+ adult wings (Fig. 2K). hh^{Mrt}/+ wings also frequently have duplicated triple row bristle patterns at the wing margin (Fig. 2L, star) typical of where L2 intersects the margin The forked L2 veins and duplicated marginal structures are consistent with the hh^{Mrt}/+ phenotype resulting from a duplicated L2 boundary rather than arising from an effect on venation per se.

A *salm* boundary is sufficient to induce L2 vein formation

To determine whether a *salm* border is sufficient to induce an L2 vein, we generated marked homozygous $salm^-$ mutant clones. The most common phenotype that we observed in such clones was a bifurcated L2 vein with the ectopic fork (2′) extending in a posterior-distal direction relative to the normal branch (2^+) (Fig. 3B,D). These ectopic L2 branches invariably formed within and along the edges of $salm^-$ clones (Fig. 3C,D,F). Forked L2 vein primordia were observed as early as the third larval instar (Fig. 3A), indicating a defect in initiating the vein pattern and that *salm* is genetically upstream of *rho*. Ectopic L2 veins were induced by either dorsal (Fig. 3B,C) or ventral (Fig. 3D,E) $salm^-$ clones, consistent with our observation that the position of the *rho* L2 stripe shifts in response to ectopic *salm* expression on either wing surface (see above). In addition, islands of wing margin structures typical of L2 veins were often observed where $salm^-$ clones intersected the margin (Fig. 3F, star). The formation of forked L2 veins in association with duplicated L2 marginal structures supports the hypothesis that a new border created by a $salm^-$ clone induces the formation of a second L2 boundary. As we have never observed loss of the endogenous L2 vein in wings containing $salm^-$ clones, other gene(s) must act in concert with *salm* to promote formation of the L2 primordium in its normal location (see Discussion).

We also observed ectopic veins branching from the L5 vein (Fig. 3B, arrow) or from the posterior cross vein. The basis for this phenotype is entirely different from that responsible for generating forked L2 veins since branched L5 veins are often composed of wild-type cells. This non-autonomy is consistent with the fact that the L5 vein forms at a significant distance from the posterior border of the *salm* expression domain. While bifurcated L5 veins are invariably associated with $salm^-$ clones near the posterior edge of the *salm* expression domain, such clones can be as much as 5-10 cell diameters removed from the ectopic L5 branch. These data suggest that the posterior boundary of the *salm* expression domain may serve as the source of a long-range vein-suppressing signal rather than the short-range vein-activating signal likely to emanate from the anterior *salm* border. The different effects of these two *salm* borders on vein formation serves as another example of the importance of the anterior versus posterior compartment context in determining the response to a given molecular event (e.g. Tabata et al., 1995; Zecca et al., 1995).

DISCUSSION

Longitudinal veins form at boundaries between discrete sectors of the wing

Two observations strongly suggest that wing veins form at the edges of discrete sectors subdividing the A/P axis of the wing. Firstly, initiation of *rho* expression in a sharp pattern of stripes implies the pre-existence of well-defined linear boundaries in the mid-third instar wing disc. Sharp lines of gene expression are often located at the interface between distinct populations of cells. For example, expression of the *single minded* gene in future ventral midline cells of the embryo occurs at the interface between mesoderm and neuroectoderm (Thomas et al., 1988). It is relevant in this context that *rho* is also expressed and functions at several known boundaries during embryogenesis such as the ventral midline and segment boundaries (Bier et al., 1990). Secondly, vein primordia form at boundaries between discrete sectors of the wing. For example, the pattern of *rho* expression in *net* or *N*; *net* double mutant wing discs consists of an alternating pattern of discrete *rho*-expressing and *rho* non-expressing domains. These domains are stably propagated throughout pupal development to generate adult flies with sectors comprising solid vein cuticle alternating with regions of purely intervein histotype. The most revealing fact about these sectors is that they are bounded by veins. Veins also serve as boundaries between alternating *salm*-expressing domains in pupal wings (see below). A similar organization of the wing disc can be inferred from mis-expression of the *iroquois* locus gene, *ara*, which gives rise to wing phenotypes specifically affecting the sectors between L2 and L3 and between L4 and L5 (Gomez-Skarmeta et al., 1996). It is tempting to compare the subdivision of the wing disc into alternating sectors to the periodic organization of the blastoderm embryo in which the pair-rule genes partition the A/P axis into stripes of different phases.

Additional support for the sector model derives from the observation that several vein primordia form at the bound-

aries of known gene expression territories. In larval wing discs, the L2 primordium forms adjacent to the anterior border of *salm* expression and *rho* expression in the L4 primordium is initiated immediately posterior to the A/P compartment boundary. For L2, the relative position of *rho*- and *salm-expressing* cells is maintained during subsequent developmental stages. During pupal development, two additional *salm* expression domains can be resolved, which abut longitudinal veins in the extreme anterior (L0) and posterior (L6) regions of the wing. In the case of L4, *rho* expression is rapidly turned off in cells contacting the A/P boundary. It is possible that the A/P border plays a direct role in initiating expression of *rho* in the L4 primordium, and that subsequent cell-cell interactions displace the position of the final L4 vein a small distance in the posterior direction, leading to the observed gap between L4 and the A/P lineage boundary in adult wings (Bryant, 1970; see Fig. 2F). The L3 primordium may also form at the edge of a domain established by a signal derived from the A/P boundary. The L3 stripe forms in close proximity to the graded anterior edge of the *ptc* RNA stripe in wing imaginal discs. β-gal staining in *ptc-GAL4*; *UAS-lacZ* individuals, which presumably is more stable than *ptc* RNA, generally abuts but does not include the L3 primordium during larval and pupal development. Cells expressing high levels of *ptc* RNA are thought to be cells with low levels of *pct* activity, since *ptc* transcription appears to be subject to a strong negative feedback from active Ptc (Capdevila et al., 1994; Li et al., 1995; Johnson et al., 1995). It has been observed that clones of ptc^- cells in the middle of the anterior compartment are surrounded by an ectopic L3 vein which comprises wild-type cells (Phillips et al., 1990; Tabata et al., 1995). Similarly, loss-of-function clones of *Protein kinase a*, which functions like Ptc to repress *ptc* expression, are encircled by ectopic veins consisting of wild-type cells (Pan and Rubin, 1995). Thus, cells with low levels of *ptc* activity may induce adjacent ptc^+ cells to assume L3 fates. Since secreted Hh is thought to be responsible for inactivating Ptc, the position of the L3 primordium might be determined by a threshold response to Hh diffusing from the posterior compartment. Consistent with secreted Hh functioning directly to determine the position of L3, we observed that the spacing between L3 and L4 was not altered as a consequence of *dpp* mis-expression (e.g. Fig. 2D).

Finally, it is interesting to note that longitudinal veins, like the well-characterized A/P and D/V compartment boundaries, have been shown to act as potent late lineage restrictions (González-Gaitán et al., 1994). Collectively, these data suggest that each longitudinal vein may be induced at the interface between distinct sectors of the developing wing primordium.

The anterior boundary of *salm* triggers formation of the L2 primordium

Direct evidence for the hypothesis that veins form at discontinuities between adjacent domains of cells derives from analysis of the L2 vein, which forms at the anterior boundary of the *salm* expression domain. Two lines of evidence support the hypothesis that this *salm* border plays an important role in determining the position of the L2 vein. The first observation is that *rho* is expressed throughout larval and pupal development in $salm^-$ cells abutting the anterior border of the central $salm^+$ expression domain. This relationship also holds in hh^{Mrt} discs and pupal wings in which the pattern of *salm* mis-expression varies greatly from one individual to another. Secondly, analysis of *salm* mosaic clones reveals that a +/– *salm* boundary in the region between L2 and L3 is sufficient for inducing an L2 vein. An important feature of these experiments is that ectopic L2 veins form within and along the edges of $salm^-$ clones where they confront $salm^+$ cells. From these data, we deduce the simple rule that an L2 vein forms within $salm^-$ cells juxtaposed to $salm^+$ cells. This vein-inducing capacity is restricted to the region between L2 and L3, however, since $salm^-$ clones in more posterior regions do not lead to bifurcated L3 or L4 veins.

We propose a model (Fig. 4F) in which *salm* specifies the location of the L2 vein primordium by an inductive mechanism at the interface between $salm^-$ and $salm^+$ cells. A well-documented instance of this type of induction is the apposition of *apterous* (*ap*)-expressing dorsal lineage compartment cells (ap^+) to ap^- ventral cells, which triggers formation of a wing margin at their interface (Díaz-Benjumea and Cohen, 1993; Tabata and Kornberg, 1994; Blair, 1993; Blair et al., 1994; Irvine and Wieschaus, 1994; Williams et al., 1994; Kim et al., 1995; Díaz-Benjumea and Cohen, 1995; Doherty et al., 1996). Analysis of ap^- clones generated in the dorsal compartment reveals that an ectopic interface between ap^+ and ap^- cells induces a ring of ectopic margin at the clone boundary. Signals passing between the dorsal and ventral compartments are polarized since cells lining the inside of the clone (ap^- cells) differentiate structures typical of ventral marginal cells, while the surrounding ap^+ cells differentiate structures associated with the dorsal margin (Díaz-Benjumea and Cohen, 1993). Response to the anterior *salm* boundary also is polarized as *rho* L2 expression is only triggered on one side of the boundary (i.e. in $salm^-$ cells). We discuss one model for generating such polarity below.

Redundant *sal* functions

Since the experiments reported in this paper were completed, a mosaic analysis of clones lacking both *salm* and a related neighboring gene (*salr*) has been reported (de Celis et al., 1996). These data suggest that the *salr* gene is a likely candidate for the gene(s) that we propose to be acting in parallel with *salm* to specify the L2 boundary since clones lacking both the *salm* and *salr* genes eliminate the endogenous L2 vein. In contrast, we never observed loss of the endogenous L2 vein in $salm^-$ single clones. To explain why $salm^-$ clone boundaries induce formation of an ectopic L2 vein when the partially redundant activity of *salr* is still present in these mutant cells, we propose that a sharp discontinuity in the level of total *sal* activity (i.e. *salm* + *salr*) is sufficient to induce an L2 boundary. In wings containing $salm^-$ clones crossing the L2 primordium, two such boundaries exist: the first in the normal location of L2 (i.e. where $salm^-$ $salr^+$ cells face $salm^-$ $salr^-$ cells), and the second lying along the $salm^-$ clone boundary (i.e. where $salm^-$ $salr^+$ cells face $salm^+$ $salr^+$ cells).

Induction of L3 fates in cells expressing low levels of *ptc* that abut cells expressing intermediate amounts of *ptc* may be another example of a modest difference in gene activity being sufficient to induce a boundary. Thus, while an ectopic L3 vein can be induced experimentally in situations where cells

expressing low levels of *ptc* confront cells strongly expressing *ptc* (Phillips et al., 1990; Tabata et al., 1995), in wild-type discs, the L3-inducing condition appears to represent a less dramatic discontinuity in *ptc* levels.

Comparison of ectopic L2 veins produced in *salm*⁻ and *sax*⁻ clones

Additional support for a model in which *salm* mediates a threshold response to Dpp signaling to define the position of L2 has been obtained by generating clones of partial loss-of-function Dpp receptor mutations (Singer et al., 1997). In these experiments, *sax*⁻ clones located between L2 and L3 induce ectopic L2 veins which run within the *sax*⁻ clones. Another similarity between *sax*⁻ and *salm*⁻ clones in this region of the wing is the formation of ectopic islands of triple row bristles where clones intersect the margin. Such *sax*⁻ clones autonomously either eliminate or reduce expression of *salm* (Singer et al., 1997). These data are consistent with the view that *salm* expression is activated by a particular threshold level of Dpp signaling and that *salm* is one of the downstream effectors of that threshold response which determines the position of the L2 boundary.

The loss of L2 in Df*salm salr* clones (de Celis et al., 1996) is similar to what is observed in *sax*⁻ clones (Singer et al., 1997). This suggests that *sax* is likely to control expression of both of these related genes. One question that arises from comparing results described in this manuscript with those of Singer et al. (1997), however, is why the forked L2 veins form at the very edge of *salm*⁻ clones, but often lie a short distance within the *sax*⁻ clones. While we do not have a definite explanation for the slight displacement of ectopic veins from the *sax*⁻ clone borders, we suspect that it is related to the observation that *sax*⁻ clones either eliminate or reduce *salm* expression depending on their position within the wing disc. *sax*⁻ clones in the anterior region of the disc most frequently lack *salm* expression (i.e. are *salm*⁻) while those in more central regions typically lead to a reduction in *salm* expression (i.e. are *salm*$^{+/-}$). Thus, in large *sax*⁻ clones, a *salm*⁻↔*salm*$^{+/-}$ border may lie a short distance within the *sax*⁻ clone. Depending on the topological arrangement of *salm*⁻ versus *salm*$^{+/-}$ cells, it is possible that a *salm*⁻↔*salm*$^{+/-}$ border running within the *sax*⁻ clone could serve as a vein-inducing boundary. Consistent with the above interpretation, Singer et al. (1996) reproducibly observed that the ectopic L2 vein formed strictly at the edge of *sax*⁻ clones at the L2 branch points where they crossed the endogenous L2 vein. The *sax* boundaries at these L2 branch points presumably lie within the region of the wing in which loss of *sax* leads to loss of *salm*. Another potential reason for the different behavior of *salm*⁻ versus *sax*⁻ clones is that Singer et al. (1996) predominantly examined large *sax*⁻ clones using the Minute technique, which typically were elongated and crossed the D-V boundary to occupy both dorsal and ventral surfaces of the wing, whereas we primarily analyzed clones restricted to one or other surface of the wing. In cases where both surfaces of the wing are *sax*⁻, dorsal-ventral induction may obscure the expected tendency of veins to strictly follow the contour of the clone. When the clone boundaries on opposite surfaces of the wing run slightly out of register, the two boundaries may compete with each other leading to unpredictable behavior of the vein (e.g. see Fig. 2C,E in Singer et al., 1997).

The position of the L2 vein can be linked to a boundary determined by the Bicoid morphogen gradient in the early blastoderm embryo

The data presented here provide a link between a boundary elaborated in response to the maternal Bicoid morphogen gradient in early blastoderm embryos and the differentiation of a linear structure along the A/P axis in adults (Fig. 4, see legend for references). Briefly, during embryogenesis, the posterior extent of zygotic *hunchback* (*hb*) gene expression is determined by a threshold response to the Bicoid morphogen gradient. The posterior edge of the *hb* domain defines the anterior limit of *even-skipped* stripe three (*eve-3*) expression, which determines the precise location of the anterior edge of *engrailed* expression in parasegment 5 (*en-PS5*). The stably inherited A/P boundary defined by *en-PS5* bisects the primordium of the wing imaginal disc. During early larval stages, *en-PS5* determines the position of a narrow stripe of *dpp* expression along the A/P boundary of the wing imaginal disc. The position of this narrow stripe of *dpp* expression within the anterior compartment in close proximity to the A/P boundary results from *en* activating expression of the short-range Hh signal and simultaneously repressing *dpp* expression in the posterior compartment. Secreted factors such as Dpp subsequently emmanate from the A/P boundary in both anterior and posterior directions. One threshold of Dpp activity determines the limits of the broader *salm* expression domain. The anterior edge of the *salm* expression domain then induces *rho* expression in adjacent cells. Finally, *rho* initiates formation of the L2 vein.

Little is currently known about the mechanism by which *salm*$^{+}$ cells might induce *rho* expression in adjacent *salm*⁻ cells. By analogy to the mechanism through which *en* activates *dpp* expression in a stripe of neighboring cells, we propose that *salm* may activate expression of a secondary short-range vein promoting signal (X) while simultaneously suppressing the response to factor X (Fig. 4F). Thus, factor X would induce neighboring *salm*⁻ cells to express *rho*, but would be unable to trigger this response in *salm*$^{+}$ cells. It remains to be determined whether the positions of other longitudinal veins and adult structures also can be linked to boundaries defined by different threshold responses to A/P boundary signals.

We thank Karen Lunde, Margaret Roark and Kathryn S. Burton for critical comments on the manuscript, and Steve Small and Mike Levine for discussions regarding the link between Eve-stripe 3 and *engrailed-P5*. We also thank Matthew Singer and William Gelbart for open and engaging discussions of their results on *sax* mutant phenotypes prior to publication. This work was supported by NSF Grant IBN-9318242, NIH Grant RO1-NS29870, Research Grant 5-FY92-1175 from the March of Dimes Birth Defects Foundation. E. B. was supported by funds from the McKnight Neuroscience Foundation, Sloan Foundation and an ACS Junior Faculty Award.

REFERENCES

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by the Hedgehog protein. *Nature* **368**, 208-214.
Bier, E., Jan, L. Y. and Jan, Y. N. (1990). *rhomboid*, a gene required for

dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogastor*. *Genes Dev.* **4**, 190-203.

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

Blair, S. S., Brower, D. L., Thomas, J. B., and Zavortink, M. (1994). The role of *apterous* in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of *Drosophila*. *Development* **104**, 713-720.

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

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signaling molecule Decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J.* **13**, 4459-4468.

Capdevila, J., Estrada, M. P., Sanchez-Herrero, E., and Guerrero, I. (1994). The *Drosophila* segment polarity gene *patched* interacts with *decapentaplegic* in wing development. *EMBO J.* **13**, 71-82.

de Celis, J. F., Barrio, R., and Kafatos, F. C. (1996). A gene complex acting downstream of *dpp* in *Drosophila* wing morphogenesis. *Nature* **381**, 421-424.

Díaz-Benjumea, F. J. and Cohen, S. M. (1993). Interaction between dorsal and ventral cells in the imaginal disc directs wing development in *Drosophila*. *Cell* **75**, 741-752.

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

Díaz-Benjumea, F. J. and García-Bellido, A. (1990). Genetic analysis of the wing vein pattern of *Drosophila*. *Wilhelm Roux's Arch. Dev. Biol.* **198**, 336-354.

DiNardo, S. and O'Farrell, P. H. (1987). Establishment and refinement of segmental pattern in the *Drosophila* embryo: spacial control of *engrailed* expression by pair-rule genes. *Genes and Dev.* **1**, 1212-1225.

Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L. and Jan, Y. N. (1996). Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation. *Genes and Dev.* **10**, 421-434.

Driever, W. and Nüsslein-Volhard, C. (1989). The Bicoid protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo. *Nature* **337**, 138-143.

Frasch, M., Warrior, R., Tugwood, J. and Levine, M. (1988). Molecular analysis of *even-skipped* mutants in *Drosophila* development. *Genes and Dev.* **2**, 1824-1838.

García-Bellido, A. (1975). Genetic control of wing disc development in *Drosophila*. *Cell Patterning* **29**, 161-178.

García-Bellido, A. (1977). Inductive mechanisms in the process of wing vein formation in *Drosophila*. *Wilhelm Roux's Arch. Dev. Biol.* **182**, 93-106.

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

Gomez-Skarmata, J. L., del Corral, R. D., de la Calle-Mustienes, E., Ferre-marco, D. and Modolell, J. (1996). *araucan* and *caupolican*, two members of the novel *iroquois* complex, encode homeoproteins that control proneural and vein-forming genes. *Cell* **85**, 95-105.

González-Gaitán, M., Capdevila, M. P., and García-Bellido, A. (1994). Cell proliferation in the wing imaginal disc of *Drosophila*. *Mech. Dev.* **46**, 183-200.

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

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

Ingam, P. W. and Fietz, M. J. (1995). Quantitative effects of hedgehog and decapentaplegic activity on the patterning of the *Drosophila* wing. *Curr. Biol.* **5**, 432-440.

Irvine, K. D. and Wieschaus, E. (1994). Fringe, a boundary specific signaling molecule, mediates interactions between dorsal and ventral cells during *Drosophila* wing development. *Cell* **79**, 595-606.

Johnson, R. L., Grenier, J. K. and Scott, M. P. (1995). *patched* overexpression alters wing disc size and pattern: transcriptional and post-translational; effects on *hedgehog* targets. *Development* **121**, 4161-4170.

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

Kornberg, T., Siden, I., O'Farrell, P. and Simon, M. (1985). The *engrailed* locus of *Drosophila*: in situ localization of transcripts reveals compartment specific expression. *Cell* **40**, 45-53.

Kühnlein, R. P., Frommer, G., Friedich, M., González-Gaitán, M., Weber, A., Wagner-Bernholz, J. F., Gehring, W. J., Jäckle, H. and Schuh, R. (1994). *spalt* encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. *EMBO J.* **13**, 168-179.

Lawrence, P. A. and Morata, G. (1976). Compartments in the wing of *Drosophila*: a study of the *engrailed* gene. *Dev. Biol.* **50**, 321-337.

Lawrence, P. A. and Struhl, G. (1982). Further studies of the *engrailed* phenotype in *Drosophila*. *EMBO J.* **1**, 827-833.

Lawrence, P. A., Johnston, P. Macdonald, P. and Struhl, G. (1987). Borders of parasegments in *Drosophila* embryos are delimited by the *fushi tarazu* and *even-skipped* genes. *Nature* **328**, 440-442.

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

Lee, J. J., von-Kessler, D. P., Parks, S., and Beachy, P. A. (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene hedgehog. *Cell* **71**, 33-50.

Li, W., Ohlmeyer, J. T., Lane, M. E., and Kalderon, D. (1995). Function of Protein Kinase A in Hedgehog signal transduction and *Drosophila* imaginal disc development. *Cell* **80**, 553-562.

Lindsley, D. L. and Grell, E. H. (1968). *Genetic variations in Drosophila melanogaster*. Carnegie Institute of Washington, Washington, D.C.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster*. Academic Press Inc., San Diego, CA.

Mohler, J. and Vani, K. (1992). Molecular organization and embryonic expression of the *hedgehog* gene involved in cell-cell communication in segmental patterning of *Drosophila*. *Development* **115**, 957-971.

Morata, G. and Lawrence, P. (1975). Control of compartment development by the *engrailed* gene. *Nature* **255**, 6147-617.

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

Noll, R., Sturtevant, M. A., Gollapudi, R. R. and E. Bier. (1994). New functions of the *Drosophila rhomboid* gene during embryonic and adult development are revealed by a novel genetic method, enhancer piracy. *Development* **120**, 2329-2338.

O'Neill, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin tagged RNA probes. *BioTechniques* **17**, 870-875.

Padgett, R. W., St. Johnson, R. D. and Gelbart, W. M. (1987). A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature* **325**, 81-84.

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

Perrimon, N. B. (1995). Hedgehog and beyond. *Cell* **80**, 517-520.

Phillips, R. G., Roberts, I. A. H., Ingham, P. W. and Whittle, J. R. S. (1990). The *Drosophila* segment polarity gene *patched* is involved in a position-signalling mechanism in imaginal discs. *Development* **110**, 105-114.

Posakony, L., Raferty, L.A. and Gelbart, W. M. (1991). Wing formation in *Drosophila melanogaster* requires *decapentaplegic* gene function along the anterior-posterior compartment boundary. *Mech. Dev.* **33**, 69-82.

Roberts, D. B. (1986). Basic *Drosophila* care and techniques. In *Drosophila*: *A Practical Approach* (ed. D.B. Roberts), pp. 1-38. Washington, D.C., IRL Press.

Sanicola, M., Sekelsky, J., Elson, S. and Gelbart, W. M. (1995). Drawing a stripe in Drosophila imaginal disks: negative regulation of decapentaplegic and patched expression by engrailed. *Genetics* **139**, 745-756.

Shroeder, C., Tautz, D., Seifert, E. and Jäckle, H. (1988). Differential regulation of the two transcripts from the *Drosophila* gap segmentation gene *hunchback*. *EMBO J.* **7**, 2881-2887.

Simpson-Brose, M., Treisman, J. and Desplan, C. (1994). Synergy between the hunchback and bicoid morphogens is required for anterior patterning in *Drosophila*. *Cell* **78**, 855-865.

Singer, M., Penton, A., Twombly, V., Hoffmann, F. M. and Gelbart, W. M. (1997). Signaling through both type I Dpp receptors is required for anterior-posterior patterning of the entire *Drosophila* wing. *Development* **124**, 79-89.

Small, S., Blair, A. and Levine, M. (1996). Regulation of two pair rule stripes by a single enhancer in the *Drosophila* embryo. *Dev. Biol.* **175**, 314-324.

Speicher, S.A., Thomas, U., Hinz, U., and Knust, E. 1994). The *Serrate* locus of *Drosophila* and its role in morphogenesis of wing imaginal discs: control of cell proliferation. *Development* **120**, 535-544.

Struhl, K. Struhl, G. and Macdonald, P. M. (1989). The gradient morphogen

bicoid is a concentration-dependent transcriptional activator. *Cell* **57**, 1259-1273.

Sturtevant, M. A. and Bier, E. (1995). Analysis of the genetic hierarchy guiding wing vein formation in *Drosophila*. *Development* **121**, 785-801.

Sturtevant, M. A., O'Neill, J. W. and Bier, E. (1994). Down-regulation of *Drosophila Egf-r* mRNA following hyperactivated receptor signaling. *Development* **120,** 2593-2600.

Sturtevant, M. A., Roark, M. and Bier, E. (1993). The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Dev.* **7**, 961-973.

Tabata, T. and Kornberg, T. B. (1994). *hedgehog* is a signalling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* **76**, 89-102.

Tabata, T., Eaton, S. and Kornberg, T. B. (1992). The *Drosophila hedgehog* gene is expressed specifically in posterior compartment cells and is a target of *engrailed* regulation. *Genes and Dev.* **6**, 2635-2645.

Tabata, T., Schwartz, C., Gustavson, E., Ali, Z. and Kornberg, T. B. (1995). Creating a *Drosophila* wing de novo, the role of *engrailed*, and the compartment border hypothesis. *Development* **121**, 3359-3369.

Thomas, J. B., Crews, S. T. and Goodman, C. S. (1988). Molecular genetics of the *single-minded* locus: a gene involved in the development of the *Drosophila* nervous system. *Cell* **52**, 133-141.

Waddington, C. H. (1940). The genetic control of wing development in *Drosophila*. *J. Genet.* **41**, 75-139.

Wagner-Berholz, J. T., Wilson, C., Gibson, G., Schuh, R. and Gehring, W. J. (1991). Identification of target genes of the homeotic gene *Antennapedia* by enhancer detection. *Genes Dev*. **5**, 2467-2480.

Williams, J. A., Paddock, S. W., Vorwerk K. and Carroll, S. B. (1994). Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature* **368**, 299-305.

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

(Accepted 2 October 1996)