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

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**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-\(\beta\) 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 hhMti 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 salm16 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-Flip; ck salm16 FRT40A/FRT40A or HS-Flip; salm16 FRT40A/FRT40A 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 (tube>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).
Another line of evidence supporting the existence of sharp borders between discrete domains of the wing imaginal 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 hhMrt imaginal wing disc. rho is expressed in an arc following the outer edge of the salm expression domain (arrow). In hhMrt 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 hhMrt 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 hhMrt 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 hhMrt 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 venal 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 hhMrt 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 hhMrt 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 dpppromoter.

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 ptc-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 hhmutant wings

We have also examined the pattern of vein primordia with respect to positional markers in wing disc clones. 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 hhmutant wings. In hhmutant 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 salmH14 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 salmH14 or the salmH16 (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 salmH14 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 salmH14 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 salmH14 clone. (F) A high magnification view of a wing margin in which a ck salmH14 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.
Figure 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; Driver 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, engrafted (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; Guilien 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 mechanisms(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 hh 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 hhMrt/+ pupal wings (Fig. 2H,J). Due to the highly variable expressivity of the hhMrt/+ 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 hhMrt/+ 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 hhMrt/+ adult wings (Fig. 2K). hhMrt/+ 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 hhMrt/+ 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 ptc 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 hhMts 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 Dfsalm 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- clones. 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 emanate 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.

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