INTRODUCTION

Wing vein development in Drosophila can be separated into two temporally distinct periods: an initiation phase, which takes place during the third larval instar when the wing primordium consists of a monolayer of cells, and a maintenance/refinement phase, which occupies prepupal and early pupal development when the wing is a bilayer of cells (Waddington, 1940; García-Bellido and de Celis, 1992; Sturtevant and Bier, 1995). In third larval instar wing discs, longitudinal vein primordia arise as a series of parallel narrow stripes of cells (Sturtevant et al., 1993). Several vein primordia lie at known boundaries between discrete sectors along the anterior-posterior (A/P) axis of the wing primordium (Sturtevant and Bier, 1995; Sturtevant et al., 1997; Lunde et al., 1998). For example, the L2 vein primordium runs along the anterior border of a broad domain of spalt-m (salm)-expressing cells (Sturtevant et al., 1997). In the case of L2, there is strong evidence that salm-expressing cells induce their neighboring salm non-expressing neighbors to become the L2 primordium (Sturtevant et al., 1997). Also, the L3 and L4 primordia form respectively along the anterior and posterior boundaries of a central stripe of cells that are engaged in Hh signaling (Sturtevant et al., 1997). These Hh-signaling cells express the patched (ptc) and decapentaplegic (dpp) genes. The posterior border of ptc expression corresponds to the A/P compartment boundary, which is the primary source of A/P patterning information in all imaginal discs. There is suggestive evidence linking the anterior border of ptc expression to formation of the L3 primordium (Phillips et al., 1990; Sturtevant et al., 1997; Strigini and Cohen, 1997), and signaling across the A/P boundary to formation of the L4 vein at the border of the posterior compartment (García-Bellido et al., 1994; Sturtevant et al., 1997).

Genes involved in initiating wing vein development in third larval instar wing discs are expressed either in narrow stripes, corresponding to vein primordia, or in broader ‘provein’ stripes, consisting of cells that are competent to become vein cells. For example, rhomboid (rho) and argos (aos) are expressed in narrow vein stripes, while Di, achaete (ac), scute (sc), caupolican (caup) and araucan (ara) are expressed in broader provein domains. rho, which encodes an integral...
membrane protein (Bier et al., 1990; Sturtevant et al., 1996), is expressed in all vein primordia and promotes vein formation throughout wing development by locally activating the EGF-R-signaling pathway (Sturtevant et al., 1993; Noll et al., 1994). $aos$ encodes an EGF-R antagonist (Freeman et al., 1992; Schweitzer et al., 1995), which feeds back negatively to inhibit EGF-R activity (Golembo et al., 1996a). $caup$ and the neighboring gene $ara$ encode related homeobox genes that promote expression of vein genes such as $rho$ and proneural genes such as $ac$ and $sc$ (Gomez-Skarmata et al., 1996). Delta ($Dl$) encodes a ligand ($Dl$) for the Notch (N) receptor (reviewed in Muskavitch, 1994; Campos-Ortega, 1995), which mediates lateral inhibitory interactions among cells in vein-competent domains during pupal development (Shellenbarger and Mohler, 1978; Parody and Muskavitch, 1993; Kooh et al., 1993).

The vein pattern is also reflected by the complementary intervein expression patterns of blistered ($bs$), which encodes the Drosophila homologue of the Serum Response Factor (DSRF) (Montagne et al., 1996) and vein ($vn$), which encodes a putative EGF-R ligand of the neuregulin/hereregulin class (Schnepf et al., 1996). $bs$ provides an essential general function for intervein development (Fristrom et al., 1994; Montagne et al., 1996) and is strongly downregulated in all vein primordia relative to intervein regions (Montagne et al., 1996). $vn$ promotes vein development and is expressed in a single strong intervein stripe running between the L3 and L4 primordia in third instar larval discs (Simcox et al., 1996).

Initiation of vein development during the third larval instar is followed by a period of vein maintenance and refinement during prepupal and pupal stages. At least three different types of cell-cell communication contribute to the refinement process (García-Bellido, 1977; Díaz-Benjumea and García-Bellido, 1990; García-Bellido and de Celis, 1992; Sturtevant and Bier, 1995): (1) lateral inhibitory signal(s) elaborated by presumptive vein cells restrict vein formation to the center of broad vein-competent domains, (2) dorsal-to-ventral signal(s) maintain vein fates in cells on the ventral surface of the wing and (3) vein continuity signal(s) promote vein formation in straight lines along the vein axis. These various signals collaborate to insure that the dorsal and ventral components of narrow veins are strictly aligned and uninterrupted.

In this manuscript, we address three major issues. First, we provide new evidence in favor of the ‘veins forming at A/P boundaries’ hypothesis. We show that expansion and contraction of the Hh-signaling domain coordinately shifts the positions of L3 vein, provein and intervein markers relative to L4, which remains tightly associated with the A/P boundary. Second, we examine the unknown regulatory relationship between genes expressed in sharp vein stripes and genes expressed in broader provein domains during the early period of vein initiation. We show that gene expression in broad provein stripes is centered over narrower stripes of gene expression in vein primordia and depends on the function of $rho$ in narrow vein stripes. We also show that different A/P boundaries organize distinct patterns of gene expression in particular veins and that crossregulatory interactions among vein, provein and intervein genes play a significant role in establishing the vein pattern. We propose a model in which boundaries between A/P sectors induce the expression of putative ‘vein-organizing’ genes in narrow vein stripes, which then orchestrate vein development in and around veins. Finally, we address the evolutionary origin of the Drosophila vein pattern. We provide evidence for the existence of a second set of vein-inducing boundaries running between vein primordia, which we refer to as paravein boundaries. We suggest that veins form at both vein and paravein boundaries in more primitive insects than Drosophila.

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 Dr Tom Kornberg (UCSF, San Francisco) for the GAL4-en stock and the en1 allele, Dr Matthew Scott (Stanford) for the UAS-ptc lines, Dr Walter Gehring (Biozentrum, University of Basel, Basel, Switzerland) for the A405.1M2 salm-lacZ enhancer trap stock, and Dr Issabel Guerrero for the UAS-hh stocks. Other stocks were obtained from the Bloomington, Indiana and Bowling Green, Ohio Drosophila Stock Centers. All crosses of GAL4 driver lines with UAS responding lines (Brand and Perrimon, 1993) were performed at 25°C.

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 and others (in Roberts, 1986) or in 50% glycerol.

In situ hybridization to whole-mount embryos or discs

In situ hybridization to whole-mount wing discs and pupal wings, carried out alone or in combination with antibody labeling, was performed with digoxigenin-labeled RNA probes (visualized as a blue alkaline phosphatase precipitate) as previously described (Sturtevant et al., 1993; O’Neill and Bier, 1994). Wings were mounted in Permoun or 50% glycerol and photographed under a compound microscope using Nomarski optics. The anti-DI antibody was kindly provided by Marc Muskavitch (Indiana University) and the anti-Bs antibody was kindly provided by Marcus Affolter (Biocenter, Basel, Switzerland).

RESULTS

Vein primordia are centered within broader vein-competent domains

To determine the precise relationships between the expression patterns of vein, provein and intervein genes, we performed a series of double-label experiments. Our primary vein marker, $rho$, is expressed in five sharp stripes 1-2 cells wide, which are likely to correspond to the primordia for the L1-L5 longitudinal wing veins (Fig. 1A). We have previously shown that neuronal precursor cells for sensory organs located along the L3 vein align with the L3 stripe of $rho$ expression in third instar wing discs (Sturtevant et al., 1993). To generalize this finding to other veins, which normally are not decorated with sensory organs, we determined the relationship between the expression patterns of $rho$ and the A101 neuronal precursor cell marker in wing primordia of $Hw^{40c}$ mutants, which have ectopic sensilla running along each longitudinal vein. Consistent with the premise that each stripe of $rho$ expression in third instar wing discs corresponds to a vein primordium, ectopic neural precursors in $Hw^{40c}$ mutants coincide with $rho$-expressing cells in third instar wing discs (data not shown) and in early everting prepupal wings (Fig. 1B).
Having confirmed that each stripe of rho expression corresponds to a longitudinal vein primordium, we determined the relative expression patterns of various genes expressed in narrow vein stripes or broader vein primordia by double-label experiments. We first compared the expression patterns of rho and Dl. In mid-to-late third instar larvae, Dl is expressed in a series of four stripes 4-6 cells wide. Double-label experiments reveal that the broader stripes of Dl protein expression are centered over the narrower L1, L3, L4 and L5 rho stripes (Fig. 1C). Additionally, double-label experiments with the anti-Dl antibody and antisense RNA probes for aos, caup and ac reveal that the three stripe of aos expression coincide with the L3, L4 and L5 Dl stripes (data not shown), that the three broad caup stripes straddle the narrower L1, L3 and L5 Dl stripes (Fig. 1E), and that the single dorsally restricted stripe of ac expression is coincident with the dorsal component of the L3 Dl stripe (Fig. 1F).

We also determined the relationship between the expression of rho and intervein markers. bs RNA and Bs protein are expressed ubiquitously in the wing pouch, but are strongly downregulated in a pattern of four stripes (Montagne et al., 1996). The L2-L5 rho stripes are centered within the troughs of Bs downregulation (Fig. 1D), which tend to be one or two cells wider than the rho stripes (e.g. there are single rows of cells flanking rho-expressing cells not expressing either rho or high levels of Bs). These data are consistent with the previous observation that L3 sensory organ precursor cells lie within the L3 trough of Bs downregulation (Montagne et al., 1996). Finally, in accordance with previously reported double-label experiments (Simcox et al., 1996), we observed that strong expression of vn is confined to the region between the L3 and L4 stripes of Dl expression (data not shown).

An important feature of these various double-labeling experiments is that the centers of all vein and provein stripes coincide. For example, the narrow stripes of rho expression run up the middle of the broader Dl stripes, and the yet broader domains of caup expression (7-8 cells wide) symmetrically straddle the odd-numbered Dl stripes. Also, as mentioned above, the stripes of Bs downregulation are centered over the narrower stripes of rho expression in veins. The nearly perfect nested registration of several vein, provein and intervein markers in third instar wing discs suggests that common positional cues coordinate expression of these genes in and around each vein primordium.

**Altering the level of Hh signaling shifts the position of the L3 primordium**

Hh is produced in the posterior compartment and diffuses a short distance into the anterior compartment where it activates target genes such as ptc and dpp in stripes running up the center of the wing disc (reviewed in Lawrence and Struhl, 1996). As mentioned previously, the L3 and L4 vein primordia form respectively along the anterior and posterior borders of the Hh-signaling domain (Sturtevant et al., 1997). In addition, a variety of evidence suggests that the anterior edge of the Hh-signaling domain defines the position of the L3 vein (Phillips et al., 1990; Sturtevant et al., 1997; Strigini and Cohen, 1997).

To investigate the relationship between the anterior border of the Hh-signaling domain and the expression of vein, provein and intervein genes, we manipulated the level of Hh and hence the width of the Hh-signaling domain. To this end, we used the GAL4/UAS system (Brand and Perrimon, 1993) to drive expression of Ptc (a Hh antagonist) or Hh in the posterior compartment. The GAL4-en driver line activates expression of UAS-transgenes in a large domain comprising the posterior compartment and in a narrow strip of anterior compartment cells (data not shown). Thus, to reduce the amount of Hh liberated from the posterior compartment, we used GAL4-en to misexpress a UAS-ptc transgene and to increase the levels of Hh, we overexpressed a UAS-hh transgene with the same GAL4 driver. Consistent with the proposition that the edges of the Hh-signaling domain define the positions of the L3 and L4

![Fig. 1. Relative positions of vein and intervein gene expression. (A) rho expression in wild-type third instar disc. In this, and subsequent panels, vein primordia L1-L5 are labeled 1-5 and the margin is indicated by M. (B) Double label for rho RNA expression (blue) and β-gal protein (brown) in a Hw^4247; A101 (neuralized-lacZ) prepupal disc. Ectopic neuralized-lacZ (neu-lacZ)-expressing neural precursors form along each rho-expressing vein primordium. The ectopic neu-lacZ-expressing cells along the L2 primordium lie just out of focus in this image. (C) Double label for rho RNA (blue) and anti-Dl protein (brown) expression in a wild-type third instar disc. Insets in C-F: magnification of the dorsal component of the L3 primordium. As double-label experiments reveal that Dl RNA and Dl protein are expressed in coincident patterns (data not shown), we conclude that the sharp stripes of rho expression run up the center of the broader Dl stripes. Double-label experiments with antisense rho and Dl digoxigenin-labeled RNA probes confirm this conclusion (data not shown). (D) Double label for rho RNA (blue) and anti-Bs (DSRF) protein (brown) expression in a wild-type third instar disc. (E) Double label for anti-Dl protein (brown) and caup RNA (blue) expression in a wild-type third instar disc. (F) Double label for anti-Dl protein (brown) and ac RNA (blue) expression in a wild-type third instar disc.](image-url)
Downregulated Bs expression in L3 also and of the vein markers Dl and there is a coordinate anterior displacement of the L3 primordium, which has an increased distance between these veins (Mullor et al., 1997; Fig. 2C). As expected from these final wing phenotypes, the width of dpp stripe is decreased in GAL4-en; UAS-pte wing discs (Fig. 2E) and increased in GAL4-en; UAS-hh discs (Fig. 2F) relative to wild-type (Fig. 2D).

In the above experiments, it was not clear whether the L3 or L4 vein was being displaced relative to the A/P compartment boundary. To address this question, we performed a series of double-label experiments from which we conclude that the position of the L3 primordium shifts when the levels of Hh signaling are manipulated, but that the L4 primordium remains tightly associated with the A/P boundary. For example, the L4 primordium forms directly along the posterior edge of the A/P compartment boundary in GAL4-en; UAS-pte wing discs (Fig. 2H) and GAL4-en; UAS-hh wing discs as it does in wild-type discs (Fig. 2G; see also Fig. 2C,E in Sturtevant et al., 1997). In these experiments, the A/P compartment boundary is marked by either the anterior border of the hh expression domain (Fig. 2G,H) or the posterior border of the dpp expression domain (Fig. 2I). These results support the view that the anterior border of the Hh-signaling domain induces formation of the L3 vein in neighboring cells.

Manipulation of Hh levels leads to the coordinate displacement of gene expression in L3

To determine whether vein and intervein markers respond in concert to alterations in the level of Hh, we performed a series of double-label experiments. In GAL4-en; UAS-pte wing discs, which have a reduced distance between the L3 and L4 vein primordia, the L3 expression patterns of Dl and rho (Fig. 3A), Dl and ac (Fig. 3B), and Dl and caup (data not shown) shift coordinately in a posterior direction. The relative positions of vein and intervein markers also are preserved in these discs as revealed by the concerted shift in the expression of rho and Bs (data not shown). Reciprocally, in GAL4-en; UAS-hh wing discs, which have an increased distance between the L3 and L4 vein primordia, there is a coordinate anterior displacement of the vein markers Dl and rho (Fig. 3C), Dl and ac (Fig. 3D), and Dl and caup (Fig. 3E). Downregulated Bs expression in L3 also shifts anteriorly in register with rho in GAL4-en; UAS-hh wing discs (Fig. 3F). As in the case of wild-type discs, the midpoints of vein and intervein stripes are coincident in GAL4-en;

Fig. 2. Shifting the position of the L3 primordium by altering the levels of Hh signaling. (A) A wild-type wing. Veins L1-L6 are labeled 1-6. (B) A GAL4-en; UAS-pte wing. Note that the L3 and L4 veins are closer to each other than in wild-type discs. The proximal section of the L4 vein is often missing in these wings (asterisk). (C) A GAL4-en; UAS-hh wing. Note that the L3 and L4 veins are spread apart relative to wild-type discs. (D) dpp RNA expression in a wild-type wing imaginal disc. Inset shows part of a wild-type wing disc double labeled for β-gal protein (brown) and hh RNA (blue) in a dpp-lacZ wing disc. (E) dpp RNA expression in a GAL4-en; UAS-pte wing imaginal disc. Inset shows part of a GAL4-en/dpp-lacZ; UAS-pte wing disc double labeled for β-gal protein (brown) and hh RNA (blue). Note that the dpp stripe is narrower than in wild-type discs, but that it abuts the posterior compartment as does the wild-type dpp stripe (compare with inset in panel D). An alternative potential explanation for the posterior shift in the L3 primordium in GAL4-en; UAS-pte flies is that elevated Ptc expression in cells lying just anterior to the A/P boundary could prevent this thin band of cells from responding to Hh (Chen and Struhl, 1996). If this were the case, there should be a gap between the A/P boundary and the domain of Hh signaling. Since the Hh target gene dpp is expressed in a narrower than wild-type stripe (Fig. 2E, compare with Fig. 2D), which none-the-less abuts the posterior compartment as normal in GAL4-en; UAS-pte flies. (F) dpp RNA expression in a GAL4-en; UAS-hh wing imaginal disc. Note that the staining is wider than that observed in wild-type discs. (G) Double label for anti-Dl (brown) and hh RNA (blue) expression in a wild-type third instar wing disc. Consistent with the broader stripes of Dl expression being centered over the narrower stripes of rho expression, the Dl L4 stripe extends 1-2 cells into the anterior compartment (inset) in contrast to the rho L4 stripe which is strictly confined to the anterior compartment (Sturtevant et al., 1997). (H) Double label for anti-Dl (brown) and hh RNA (blue) expression in a GAL4-en; UAS-pte third instar wing disc. (I) Double label for anti-Dl (brown) and rho RNA (blue) expression in a dpp-lacZ; GAL4-en; UAS-hh third instar wing disc. Inset: magnification of the ventral component of the L4 primordium.
UAS-\textit{ptc} and GAL4-\textit{en}; UAS-\textit{hh} wing discs. Cumulatively, these data suggest that gene expression in and around the L3 primordium is organized by a single positional cue specified by the anterior limit of the Hh-signaling domain.

In addition to the coordinate displacement of Dl and \textit{rho} expression in GAL4-\textit{en}; UAS-\textit{ptc} wing discs (Fig. 3A), we observed that the levels of Dl and \textit{rho} expression in L4 are significantly reduced relative to wild type (Figs 2H, 3A) and that adult wings generated from these discs often have gaps in the L4 vein (Fig. 2B, asterisk). This reduction in Dl and \textit{rho} expression in L4 suggests that the Hh-signaling domain in the anterior compartment normally sends a signal to adjacent posterior compartment cells to initiate L4 formation. Since fewer cells would express this putative L4-inducing signal in GAL4-\textit{en}; UAS-\textit{ptc} wing discs than in wild-type discs, the level of signal might fall below that required to reliably induce L4 formation. In accord with this possibility, loss of \textit{vn} function in the anterior compartment non-autonomously leads to loss of the L4 vein (Garcia-Bellido et al., 1994). This non-autonomous effect on L4 formation and the observation that the L4 primordium invariantly abuts the A/P boundary reinforces the view that signaling across the A/P boundary induces the formation of L4.

\section*{Crossregulation between vein and intervein genes consolidates vein boundaries}

Since the expression of vein, provein and intervein genes is initiated almost simultaneously in third larval instar wing discs, it is possible that crossregulatory interactions among these early acting genes, as well as continued signaling from boundaries, are important for establishing the vein pattern. To address this question, we examined the expression of vein, provein and intervein genes in early acting vein mutants (Fig. 4), which have been previously shown to disrupt initiation, rather than maintenance, of vein development (Sturtevant et al., 1995; Gomez-Skarmata et al., 1996). Early acting loss-of-vein mutants include the recessive mutants \textit{rho}^{\textit{ve}}, a cis-regulatory allele of \textit{rho} that lacks detectable \textit{rho} expression in vein primordia (Fig. 4A), \textit{vn}^{\textit{L}} (Fig. 4B), \textit{rho}^{\textit{ve}} \textit{vn}^{\textit{L}} double mutants (Fig. 4C), \textit{inr}_{\textit{DFM}} (which behaves as an L3-specific loss-of-function allele of the \textit{iro} locus and does not survive to adulthood), \textit{radius incompletus} (\textit{ri}), which is a likely regulatory allele of the \textit{knirps/kniirps-related} locus (Lunde et al., 1998) (Fig. 4D) and \textit{abrupt} (\textit{ab}) (Fig. 4E).

We also examined expression of markers in early acting extravein mutants such as the recessive \textit{net} mutant (Fig. 4F) and the dominant \textit{rho}^{\textit{St}} enhancer piracy line (Noll et al., 1994; Fig. 4H).

The results of analyzing the initial expression patterns of vein and intervein genes in early vein mutants are tabulated in Table 1 and summarized schematically in Fig. 9. Examples of these crossregulatory interactions are presented in Fig. 5. Two major conclusions can be drawn from these results. First, crossregulatory interactions do play a significant role in establishing the initial sharp vein-versus-intervein pattern. For example, in \textit{vn}^{\textit{L}} wing discs, which lack detectable expression of the EGF-R ligand \textit{vn} (Simcox et al., 1996), expression of Dl (Fig. 5A) and \textit{rho} (Sturtevant and Bier, 1995) is virtually eliminated in the L4 primordium. \textit{rho}-mediated activation of EGF-R signaling also contributes to establishing the vein pattern since both the L3 and L4 stripes of Dl expression are severely compromised in \textit{rho}^{\textit{ve}} \textit{vn}^{\textit{L}} double mutants (Fig. 5B). \textit{rho} and \textit{vn} also collaborate to activate \textit{ac} and \textit{sc} expression in the L3 primordium as expression of \textit{ac} and \textit{sc} in broad L3 stripes is lost in \textit{rho}^{\textit{ve}} \textit{vn}^{\textit{L}} double mutants discs (Fig. 5C-D). The presence of isolated \textit{sc}-expressing cells in \textit{rho}^{\textit{ve}} \textit{vn}^{\textit{L}} discs (Fig. 5D, arrows), likely to be L3 sensory organ precursors, may explain why L3 sensilla are usually present in \textit{rho}^{\textit{ve}} \textit{vn}^{\textit{L}} wings (Diaz-Benjumea and Garcia-Bellido, 1990). Finally, \textit{rho} function is necessary and sufficient for initiating \textit{argos} expression throughout the wing disc (Table 1).

The \textit{iro} locus is known to play a central role in establishing the vein pattern in odd-numbered veins. For example, the \textit{inr}_{\textit{DFM}} mutation causes severe reduction in \textit{rho} (Gomez-Skarmata et al., 1996), \textit{ac} (Gomez-Skarmata et al., 1996) and Dl (Fig. 5E) expression in the L3 primordium. Interestingly, however, the pattern of Bs downregulation in L3 is normal in \textit{inr}_{\textit{DFM}} mutant discs (Fig. 5F). This wild-type expression of Bs contrasts with the weakened pattern of Bs downregulation in \textit{rho}^{\textit{ve}} \textit{vn}^{\textit{L}} double

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Effects of shifting the anterior border of the Hh-signaling domain on expression of vein and intervein genes in the L3 primordium. (A) Dl (brown) and \textit{rho} (blue) expression in a GAL4-\textit{en}; UAS-\textit{ptc} third instar wing disc. Note that the L3 and L4 vein primordia are closer together than in wild-type discs and that Dl and \textit{rho} expression in L3 shift in register. (B) Dl (brown) and \textit{ac} (blue) expression in a GAL4-\textit{en}; UAS-\textit{ptc} third instar wing disc also shift together posteriorly. (C) Dl (brown) and \textit{rho} (blue) expression in a GAL4-\textit{en}; UAS-\textit{hh} third instar wing disc. Note that the L3 and L4 vein primordia are spread apart and that Dl and \textit{rho} expression in L3 shift in register. (D) Dl (brown) and \textit{ac} (blue) expression in a GAL4-\textit{en}; UAS-\textit{hh} third instar wing disc also shift anteriorly together. (E) Dl (brown) and \textit{caup} (blue) expression in a GAL4-\textit{en}; UAS-\textit{hh} third instar wing disc are coordinately displaced. (F) Bs (brown) downregulation and \textit{rho} (blue) expression in the L3 primordium of a GAL4-\textit{en}; UAS-\textit{hh} third instar wing disc also shift anteriorly in register.}
\end{figure}
mutant wing discs (Fig. 5G). Bs expression in intervein regions is partially dependent on net function since the area of Bs downregulation in net mutant wing discs is enlarged (Fig. 5H, brackets) in regions corresponding to those ectopically expressing rho (compare with Fig. 6A).

The second major point regarding crossregulatory interactions among vein, provein and intervein genes is that individual stripes of gene expression may represent independent units of regulation. This point is most obvious for the ri and ab mutants in which expression of all relevant vein, provein and intervein markers (e.g. downregulated Bs expression) is strictly dependent on ri function in L2 and on ab function in L5. The distinct behaviors of the L3, L4 and L5 Di stripes in vn1 versus rho ve vn1 mutants described above is another example of stripe-dependent regulation of gene expression. The differential requirement for EGF-R signaling to activate expression of genes in particular veins presumably reflects differing threshold requirements for EGF-R signaling.

Analysis of plexate versus solid ectopic vein phenotypes

The mutant analysis discussed above raises an apparent paradox. The rhoSld mutant has intervein sectors converted into solid veins (Fig. 4H), while net mutants have a reticulum of ectopic veins of normal thickness running through similar regions of the wing (Fig. 4F). The pattern and duration of ectopic rho expression in net (Fig. 6A) and rhoSld (Fig. 6C) third larval instar wing discs and prepupal wings (data not shown) are very similar, however. A clue to the basis for the different adult vein phenotypes of rhoSld versus net flies is

Table 1. Expression of vein and intervein genes in early acting vein mutants

<table>
<thead>
<tr>
<th>Gene</th>
<th>ri1</th>
<th>iro</th>
<th>ab1</th>
<th>rho ve</th>
<th>vn1</th>
<th>rho ve vn1</th>
<th>net</th>
<th>rho Sld</th>
</tr>
</thead>
<tbody>
<tr>
<td>caup</td>
<td>NA</td>
<td>–L3</td>
<td>–L5</td>
<td>wt</td>
<td>wt</td>
<td>–L3</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>ac</td>
<td>NA</td>
<td>–L3</td>
<td>NA</td>
<td>wt</td>
<td>wt</td>
<td>–L3</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>sc</td>
<td>NA</td>
<td>–L3</td>
<td>NA</td>
<td>wt</td>
<td>wt</td>
<td>↓L3</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>Bs</td>
<td>filled L2</td>
<td>wt</td>
<td>filled L5</td>
<td>wt</td>
<td>wt</td>
<td>filled L2</td>
<td>↓L2-L3</td>
<td>±ect. L4:L5</td>
</tr>
<tr>
<td>vn</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>–IV.3</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
</tbody>
</table>

The expression of genes expressed in vein and intervein patterns (left-most vertical column) was examined in various mutant genetic backgrounds (top horizontal row). Entries in the table indicate whether the expression pattern of a given gene is wild-type (wt) or abnormal in a particular mutant. Examples of symbols indicating abnormal gene expression are as follows: –L3, L3 expression is lost; –IV.3, expression of vn in the intervein region between L3 and L4 in third instar wing discs is lost; ↓L2-L4, L2 expression is reduced; ect.L2:L3, ectopic veins form in between L2 and L3; filled L2, downregulated expression of Bs in L2 is not observed (i.e. vein expression = intervein expression); ±L3, L4 = downregulated expression of Bs in L3 and L4 is less pronounced than in wild-type discs; NA = not applicable (e.g. the gene is normally not expressed in a vein affected by the particular mutant in question). Bold entries are shown as data in figures.

Fig. 4. Adult wings of early acting wing vein mutants. (A) A rho ve / rho ve adult wing. (B) A vn1/vn1 adult wing. (C) A rho ve vn1/rho ve vn1 adult wing. (D) An ri1/ri1 adult wing. (E) An ab1/ab1 adult wing. (F) A net/net adult wing. Solid arrows indicate the P1 and P6 paraveins and the open arrow points to the P2 paravein. In other net wings, veins can be found in all paravein positions (i.e. P1-P6). (G) A N+/netnet adult wing. Brackets indicate domains of solid ectopic veins between L2 and L3 and between L4 and L5. Note the absence of ectopic veins between L3 and L4. (H) A rhoSld/+ adult wing. Note that ectopic veins (brackets) form in the same position as in N+/netnet wings (G).
provided by N+/net double mutants (Díaz-Benjumea et al., 1990; Sturtevant et al., 1995; Fig. 4G) in which lateral inhibition also is compromised. N+/net flies and rho^Stl^ flies have solid ectopic veins forming throughout the same intervein sectors (Fig. 4G, brackets – compare with Fig. 4H), suggesting that the net single mutant phenotype results from the ectopic activation of two competing genetic pathways: a vein promoting pathway and a lateral inhibitory pathway which restricts vein formation.

Because Dl encodes a ligand for N and is expressed early in vein competent domains, we examined the pattern of Dl expression in net mutant discs. Consistent with Dl playing a role in limiting vein formation in net mutants, Dl RNA (data not shown) and Dl protein (Fig. 6B) are mis-regulated much like rho in net mutant wing discs (compare brackets in Fig. 6A,B). In contrast, Dl RNA (data not shown) and Dl protein (Fig. 6D) are expressed relatively normally in rho^Stl^ wing discs (compare with Fig. 6B). Thus, if we assume that ectopic expression of rho in the absence of effective lateral inhibition (e.g. as in rho^Stl^ flies or N+/net double mutants) generates solid ectopic vein phenotypes, the coordinate mis-expression of rho and Dl in net discs and the solitary mis-expression of rho in rho^Stl^ discs could account for the different resulting vein phenotypes of net versus rho^Stl^ mutants. The observation that genes such as caup and ac are expressed normally in net mutant discs reinforces the view that the net phenotype results from the selective coordinate mis-expression of rho and Dl.

Cryptic ‘paraveins’ run between vein boundaries

A variety of evidence indicates that biologically meaningful boundaries also run between and parallel to longitudinal vein primordia. We refer to these cryptic borders as paravein boundaries since ectopic veins (paraveins) have a strong tendency to form in these positions in a variety of extravein mutants (Fig. 4F; arrows; Thompson, 1974). Four likely paravein boundaries (P2, P4, P5 and P6; see Fig. 7J) can be observed in third instar wing discs. The position of the putative P4 paravein between the primordia for L3 and L4 can be revealed by a stripe of rho mis-expression in fused mutant wing discs (Fig. 7A). P4 also is marked by a short ectopic vein between L3 and L4 in various extravein mutants (Sturtevant et al., 1993; Sturtevant and Bier, 1995; see below) and a true vein, which is found in this position in primitive insects, forms along the anterior boundary of en expression (see below; Fig. 7H). The proposed P5 paravein boundary runs between the primordia for the L4 and L5 veins in the approximate location of the posterior border of the spalt expression domain in third instar discs (Fig. 7B; Sturtevant et al., 1997). In pupal wings, it is unambiguous that P5 borders the posterior edge of salm expression. Thus, a short ectopic section of vein (P5) running between L4 and L5 in net/+ adult wings (Fig. 7C) can be visualized in net/+ pupal wings as an ectopic segment of Dl expression abutting the posterior edge of the spalt domain (Fig. 7D). The positions of the P2 and P6 paraveins also are likely to be defined in third instar discs as revealed by ectopic expression of rho in net mutants. In mid-third instar wing discs, ectopic rho expression is bounded by L2 anteriorly and by L5 posteriorly (Fig. 6A; Sturtevant and Bier, 1995). Shortly thereafter in late third instar discs, however, rho expression expands anteriorly beyond L2 and posteriorly behind L5 (data not shown). These enlarged borders of rho mis-expression in net discs are likely to correspond to the positions of the P2 and P6 paraveins.

Fig. 5. Crossregulatory interactions among early acting vein and intervein genes. (A) Dl expression in a vn^1 third larval instar disc. The intensity of the L4 stripe is reduced (arrow) relative to wild-type and to expression in other veins in the same disc. Similar results were obtained using a Dl RNA probe indicating that the loss of expression occurs at the level of transcription. (B) Dl expression in a rho^ve vn^1 double mutant third larval instar disc. Note that expression in the L3 and L4 stripes is greatly reduced (arrows). Similar results were obtained using a Dl RNA probe. (C) ac in expression in a rho^ve vn^1 third larval instar disc is lost in the L3 stripe (bracket). (D) sc in expression in a rho^ve vn^1 third larval instar disc is lost in the broad L3 stripe, but is retained in isolated cells likely to be sensory organ precursor cells (the solid arrow indicates a cell in the focal plane of the image and the dotted arrow indicates two cells in an adjacent focal plane). (E) Dl expression in an iro^DFM2/iro^DFM2 third larval instar disc. Note that expression in the L3 primordium is severely reduced (arrow). iro^DFM2/iro^DFM2 homozygous larvae were identified from an iro^DFM2/TM6,Ub balanced stock based on the absence of the Tb marker. (F) Bs expression in an iro^DFM2/iro^DFM2 third larval instar disc is indistinguishable from wild-type. (G) Bs expression in a rho^ve vn^1 third larval instar disc. Note that Bs downregulation in L2 is undetectable (arrowhead) and that downregulation in L3 and L4 is significantly less pronounced than in wild-type (arrows; compare with Fig. 5F). Bs expression in the sector between L3 and L4 also may be elevated relative to wild-type. (H) Bs expression in a net/net third larval instar disc. The area of Bs downregulation, particularly anterior to L3 and posterior to L4 (brackets), is expanded relative to wild-type (compare with Fig. 5F).

Boundaries in the Drosophila wing imaginal disc
Fig. 6. DI and rho are mis-expressed in net mutant discs. (A) rho expression in a net third larval instar disc. rho is mis-expressed in solid sectors (brackets) between L2 and L3 and between L4 and L5, but is excluded from the intervein region between L3 and L4. (B) DI expression in a net third larval instar disc. DI is mis-expressed in solid sectors (brackets) similar to rho (see A). DI RNA expression is similarly mis-regulated in net mutant discs, indicating that ectopic expression occurs at the level of transcription. (C) rho transgene expression in a rho

The P4 and P5 paraveins also can be marked by rows of ectopic bristles in wings of AS-C

DISCUSSION

Boundaries along the A/P axis initiate vein formation

The data presented in this study reinforce the view that wing veins form at boundaries between discrete sectors in the wing (Sturtevant and Bier, 1995; Sturtevant et al., 1997). When the location of a vein-inducing boundary is altered experimentally, the positions of vein markers shift correspondingly in register (Sturtevant et al., 1997; this study). For example, when the anterior edge of the salm expression domain is altered, the position of the L2 shifts correspondingly (Sturtevant et al., 1997). Because the domain of salm expression is determined by a threshold response to Dpp emanating from a stripe of cells running along the A/P compartment boundary (Nellen et al., 1996; LeCuit et al., 1996; Lawrence and Struhl, 1996; Singer et al., 1997), the position of L2 depends most directly on Dpp activity. In contrast to L2, the positions of the L3 and L4 veins are likely to be determined primarily by Hh signaling along the A/P compartment boundary rather than by Dpp (Sturtevant et al., 1997; Strigini and Cohen, 1997). Consistent with this hypothesis, the positions of several L3 vein and provein markers shift concertedly in response to displacement of the anterior edge of the Hh-signaling domain. These experiments also lend further support to the proposal that the L4 vein forms along the anterior border of the posterior compartment in response to a signal such as Vn produced in the anterior compartment. An important invariant in wild-type and mutant discs is that narrow vein stripes run through the centers of broader domains of provein gene expression and downregulated expression of intervein genes.

A hierarchical model for vein formation at boundaries

Four classes of models for activating gene expression in narrow versus broad stripes can be entertained (Fig. 8). In the first model (Fig. 8A), crude A/P patterning information activates gene expression in large A/P sectors such as the anterior and posterior compartments. In analogy to embryonic segmentation, genes expressed in these large A/P sectors could collaborate to activate gene expression in more restricted provein stripes, which then refine to the final sharp vein pattern. During later pupal stages, veins are indeed selected from boundaries in the pupa (Fig. 7G,H; small arrow and arrowhead) suggesting that late en boundaries organize various linear features of the adult wing.
competent patterns of $Dl$, $ac$ and $sc$ expression and $Bs$ downregulation depend on $rho$, which is expressed in narrow stripes along well-defined A/P boundaries. Finally, expression of $Dl$, $ac$, $sc$ and $rho$ is initiated contemporaneously and not according to a sequence of broad to narrow stripes. We cannot, however, rule out the possibility that a progressive refinement mechanism leads to the pattern of $caup$ and $ara$ expression in the odd-numbered vein primordia (Gomez-Skarmata et al., 1996; Gomez-Skarmata and Modolell, 1996).

In the second model, A/P patterning generates sharp borders between discrete sectors of the wing disc. These sharp borders serve as sources for locally acting signals that function in a dose-dependent fashion to activate expression of vein genes in narrow vein stripes and expression of provein genes in broader

---

Fig. 7. Paravein boundaries run between vein primordia. (A) $rho$ expression in a $fu^1$ mutant wing disc. $fu^1$ adult wings have L3 and L4 shifted closer together. The arrow indicates the location of a stripe of $rho$ expression running between L3 and L4. Similar adult and early pupal phenotypes are observed in $knot$ mutants, which often have a vein segment running between L3 and L4 (data not shown). (B) The pattern of lacZ expression driven from a $sal-lacZ$ enhancer trap insertion (brown) relative to $rho$ expression in veins (blue). The anterior boundary of this staining territory abuts L2 and the posterior edge falls half way between the primordia of L4 and L5. This latter boundary is in the approximate position expected for paravein P5. (C) A $net^+/+$ adult wing. The arrow indicates an ectopic P5 ‘paravein’ which frequently forms in this location. (D) $sal-lacZ$ (brown) and $Dl$ (blue) expression in developing veins of a $net^+/+$ heterozygous pupal wing. $Dl$ expression in the P5 paravein (arrow) forms at the posterior edge of the $spalt$ domain. (E) A $h^1 rho^{osl} rho^{osl}$ adult wing. Arrows indicate rows of ectopic bristles running between longitudinal veins along the putative P4 and P5 boundaries. (F) A pupal $h^1 rho^{osl} rho^{osl}$ wing double stained for En (mAb4D9) and mAb22C10 (mAb22C10 labels differentiating neurons). Neurons form at the anterior edge of the En domain (arrows). En expression is also downregulated in posterior compartment veins (marked 4 and 5). (G) An adult syrphid fly wing has a vein in the P4 position (large arrow). Other morphological features of the wing include a double vein corresponding to P6 (small arrow) and a wedge shaped fold in the wing (arrowhead). (H) A pupal syrphid wing stained with Mab4D9 (Mab4D9 cross-reacts with En proteins in various insect species). The anterior boundary of En expression borders the P4 vein (large arrow). En also is sharply downregulated in posterior veins and sharp En boundaries in the pupal wing mark other morphological features of the adult wing form (small arrow and arrowhead – compare with G). (I) An adult cranefly wing has veins in locations corresponding to Drosophila paraveins as well as veins. (J) Diagram of an archetypal wing vein pattern hypothesized to be ancestral to diverse modern insects (redrawn from Colless and McAuliffe in The Insects of Australia). We propose a correspondence between our nomenclature for alternating veins and paraveins (L0-L6 and P0-P7) and the standard nomenclature for the primitive vein pattern (in parentheses).
vein-competent domains (Fig. 8B). Activation of Hh responsive genes in the anterior compartment in broad stripes (e.g. dpp) versus narrow stripes (e.g. ptc) along the A/P compartment border is an example of this kind of mechanism (Strigini and Cohen, 1997). According to this model, genes expressed in broad vein-competent domains are activated by low to high levels of this signal, while genes expressed in narrow vein primordia are activated only by high levels of signal. One prediction of this model is that broad and narrow stripes of gene expression should all be in register along one sharp border. As we observe that vein-competent domains are centered precisely over narrower stripes of rho expression in vein primordia in wild-type and mutant discs, we do not favor this model either.

In the third model, signals passing between adjacent A/P sectors activate expression of ‘vein-organizing genes’ in sharp vein stripes (Fig. 8C). These vein-organizing genes activate expression of secondary short-range signals that activate expression of provein genes in broader vein competent domains. Diffusion of Dpp from a narrow stripe of cells along the A/P compartment boundary of the wing disc to activate salm expression in a broad central sector centered over dpp-expressing cells is an example of this type of symmetrical inductive mechanism (Nellen et al., 1996, Lecuit et al., 1996). According to this model, sharp vein stripes should run up the middle of broader provein domains rather than along one edge of them. Another consequence of the third model is that gene expression in broad provein domains would be disrupted in mutants failing to produce the secondary signal. This model is consistent with double-label experiments showing that provein domains are centered over narrow vein primordia and with the observation that expression of provein genes such as Dl, ac and sc are dependent on rho, which is expressed in narrow stripes. The non-autonomous action of rho to influence fates of neighboring cells has also been observed in a variety of other developmental contexts (García-Bellido, 1977; Golembo et al., 1996b; Bier, 1998; Guichard et al., unpublished data).

Finally, a fourth model is that genes that are initially expressed imprecisely in vein primordia, in provein and in intervein domains crossregulate to establish the sharp vein pattern. In this study, we show that several crossregulatory interactions are indeed important in establishing the pattern of vein-versus-intervein expression of other genes. For example, rho and vn, which promote EGF-R activity, are required for the proper expression of Dl, ac, sc and bs (a complete summary of the observed crossregulatory interactions is presented schematically in Fig. 9). Furthermore, strong expression of dominant negative and activated forms of EGF-R pathway components alters gene expression in vein primordia (Roch et al., 1998). Another example of coordinated regulation is the suppression of rho and Dl expression by net in alternating intervein sectors of the wing. One implication of net functioning as a negative regulator of both rho and Dl is that these two opposing genes may form a meaningful genetic submodule during vein development. Similar mechanisms have been shown to underlie patterning of the nervous system in which proneural genes both promote neural development within broad competent domains and activate genes required for lateral inhibition, which restrict the number of cells assuming neuronal fates (Hinz et al., 1994; Signson et al, 1994).

In our favored model (Figs 8C,D, 9), boundaries between discrete sectors along the A/P axis initiate expression of vein-organizing genes, which in turn orchestrate expression of various genes in and around veins. A mutant in a vein-organizing gene should lack any hint of vein, provein or intervein features in the position of that vein primordium in third instar larvae. Candidate mutants in vein-organizing genes are ri for the L2 primordium and ab for the L5 primordium. Additional evidence that ri functions as a vein-organizing gene is that this mutation is likely to be a regulatory allele of the knirp/knirps-related locus, which specifically eliminates expression of the knirp and knirps-related transcription factors in a narrow stripe of cells corresponding to the L2 primordium (Lunde et al., 1998). The coordinate regulation of vein, provein...
Fig. 9. Model for initiating and maintaining vein development on a vein-by-vein basis. We propose that vein formation is initiated at boundaries between discrete A/P sectors of the wing disc (indicated in blue type). The vein-inducing boundary for the L2 primordium is likely to be the border between salm-expressing and salm non-expressing (or weakly expressing) cells (Sturtevant et al., 1997). The L2 primordium forms within the salm non-expressing domain of cells. The vein-inducing boundary for the L3 primordium may be the border between Hh responding cells expressing high/moderate levels of ptc and cells expressing very low levels of ptc. The L3 primordium forms within the domain of very low ptc expression (Phillips et al., 1990; Sturtevant et al., 1997). With respect to the L4 primordium, the vein-inducing boundary is likely to be the A/P compartment boundary itself. Although the L4 vein is displaced posteriorly by a few cell diameters from the A/P compartment boundary in adult flies, the L4 primordium initially abuts the A/P boundary in third instar wing discs (Sturtevant et al., 1997; this study). Currently, there is not a good candidate border known in the position of the L5 primordium. Vein-inducing boundaries might act directly to regulate gene expression in and around vein primordia, or might act through intermediate vein-organizing genes (indicated in red type) to orchestrate gene expression. Mutants lacking the function of a vein-organizing gene should lack expression of all vein markers and should not downregulate expression of intervein markers in that vein. Based on this criterion, candidate vein-organizing genes are ri for the L2 vein and ab for the L5 vein. For further evidence that ri functions as a vein-organizing gene see Lunde et al. (1998). Whether there are similar genes acting to organize gene expression in L3 and L4 remains to be determined. As depicted in Fig. 8C, we propose that vein-inducing boundaries and/or vein-organizing genes activate expression of vein genes (e.g. rho) in narrow stripes, initiate the production of locally acting signals that activate gene expression in broader vein-competent regions centered over veins (e.g. DI and ac/sb) and suppress expression of intervein genes (e.g. bs). These genes then engage in various vein-specific crossregulatory interactions (indicated in black type). 

Symbol key: Arrows in the diagram, activating interactions; barred lines, suppressive interactions; LV.1-LV.5, intervein regions; L1-L5, longitudinal vein primordia; the thick red line labeled A/P, the anterior-posterior compartment boundary.

and intervein genes in L3 suggests that there also may be a vein-organizing gene for L3. This stripe-by-stripe form of gene regulation in veins is reminiscent of the independent regulation of primary pair-rule genes by distinct stripe-specific enhancer elements. The restricted pattern of rho mis-expression in net mutant discs suggests that intervein sectors also may represent autonomous domains of gene expression. Identification and analysis of cis-regulatory sequences driving gene expression in multiple veins will be required to determine whether independent enhancer subelements control gene expression in individual veins.

According to our preferred model, boundaries in third instar wing discs induce gene expression in narrow vein primordia, which produce short-range signal(s) to activate expression of genes in broader provein domains. Subsequently, veins are selected by lateral inhibition from the broad vein-competent domains during pupal development. It may seem counterintuitive to generate broad provein stripes from narrow stripes and then refine them to narrow stripes again. This is not the case, however, since the period of vein initiation and vein refinement represent two very different developmental contexts. During vein initiation, the developmental task is to define the locations of vein primordia and to center these veins crudely within broader vein competent domains and regions of intervein gene downregulation. In contrast, during pupal development, dorsal-→ventral inductive signals and lateral inhibition within broad vein-competent domains collaborate to assure that the dorsal and ventral components of veins on the two surfaces of the wing become precisely aligned. Thus, early patterning events provide an approximate location for veins and subsequent refinement processes, such as lateral inhibition, adjust the fine positions of the dorsal and ventral components of veins with respect to one another.

**Paravein boundaries in Drosophila** are likely sites of vein formation in primitive insects

Boundaries such as the posterior salm border run between existing vein primordia in third instar larvae and early pupae. In various wing vein mutants, ectopic veins, which we refer to as paraveins, preferentially form at these mid-intervein locations (Thompson, 1974). In primitive insects, which have up to twice the number of veins as *Drosophila*, it is likely that vein development is initiated along boundaries corresponding to paraveins as well as veins (Fig. 7I). A possible relationship between veins and paraveins in *Drosophila* and the vein pattern in primitive insects is presented in Fig. 7J. According to this model, veins form at all vein and paravein positions in primitive insects. Vein patterns in insects with fewer than the full complement of veins generally have been interpreted by assuming that ancestral veins are fused into a reduced number of veins (e.g. R2 and R3 being fused to generate L2 in *Drosophila*) (e.g. see Colless and McAlpine, 1970; Nijhout,
We thank Raffi Aronian, Dan Lindsay, Karen Lunde, Margaret Roark and the helpful reviewers for comments on the manuscript. This work was supported by NIH Grant No. RO1-NS29870-01, NSF #IBN-9318242 and NSF #IBN-9604048.

REFERENCES


Boundaries in the *Drosophila* wing imaginal disc


