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blistered: a gene required for vein/intervein formation in wings of *Drosophila*

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SUMMARY

We have characterized the *blistered (bs)* locus phenotypically, genetically and developmentally using a set of new *bs* alleles. Mutant defects range from wings with ectopic veins and intervein blisters to completely ballooned wings where the distinction between vein and intervein is lost. Mosaic analyses show that severe *bs* alleles behave largely autonomously; homozygous patches having vein-like properties. Developmental analyses were undertaken using light and electron microscopy of wild-type and *bs* wings as well as confocal microscopy of phalloidin- and laminin-stained preparations. *bs* defects were first seen early in the prepupal period with the failure of apposition of dorsal and ventral wing epithelia. Correspondingly, during definitive vein/intervein differentiation in the pupal period (18-36 hours after puparium formation), the extent of dorsal/ventral reapposition is reduced in *bs* wings. Regions of the wing that fail to become apposed differentiate properties of vein cells; i.e. become constricted apically and acquire a laminin-containing matrix basally.

To further understand *bs* function, we examined genetic interactions between various *bs* alleles and mutants of two genes whose products have known functions in wing development. (i) *rhomboid*, a component of the EGF-R signalling pathway, is expressed in vein cells and is required for specification of vein cell fate. *rho^ve* mutations (lacking rhomboid in wings) suppress the excess vein formation and associated with *bs*. Conversely, *rho* expression in prepupal and pupal *bs* wings is expanded in the regions of increased vein formation. (ii) The integrin genes, *inflated* and *myospheroid*, are expressed in intervein cells and are required for adhesion between the dorsal and ventral wing surfaces. Loss of integrin function results in intervein blisters. Integrin mutants interact with *bs* mutants to increase the frequency of intervein blisters but do not typically enhance vein defects. Both developmental and genetic analyses suggest that the *bs* product is required during metamorphosis for the initiation of intervein development and the concomitant inhibition of vein development.

Key words: laminin, *rhomboid*, integrin, veinlet, *myospheroid*, *inflated*, wing morphogenesis, *Drosophila*

INTRODUCTION

The wings of *Drosophila* are derived from relatively simple epithelial bilayers that have proven useful for studying many aspects of development (Blair, 1994; Couso et al., 1994; Diaz-Benjumea and Hafen, 1994; Garcia-Bellido and De Celis, 1992; Schubiger and Palka, 1987; Willliams et al., 1993). Non-neural wing tissue is organized into a patterned distribution of two cell types, intervein (90%) and vein (10% of the wing surface). Each non-neural cell forms an apical cuticular hair so hair density is an indicator of cell surface area (Dobzhansky, 1929). Intervein regions consist of large cells (low hair density) whose major function is to provide an aerodynamic surface. As the fly ecloses intervein cells die leaving a double layer of transparent, hair-studded cuticle (Johnson and Milner, 1987). A reproducible pattern of five longitudinal veins (designated L1 to L5 from anterior to posterior) and two cross veins traverses the cuticular sheet. Veins consist of narrow channels lined by small living cells that provide structural support for the wing and passage for neurons and trachea.

During wing development intervein cells are responsible for connecting and holding the two surfaces of the wing together (Fristrom and Fristrom, 1993; Waddington, 1941). To that end, intervein cells differentiate a highly specialized system of cytoskeletal supports, the transalar array (Tucker et al., 1986), anchored in integrin-mediated basal adhesions (Fristrom et al., 1993). Vein cells, in contrast, are relatively unspecialized; they do not form connections with the opposite surface, do not express integrin and do not differentiate transalar arrays. We show here that vein cell differentiation involves an apical constriction leading to the characteristic small cell size and the acquisition of a laminin-containing basal matrix.

Numerous mutations that affect the pattern of wing veins have been isolated (Lindsley and Zimm, 1992). Many of these mutants have been classified according to their loss-of-function phenotypes (Diaz-Benjumea and Garcia-Bellido, 1990;

Garcia-Bellido and De Celis, 1992) such as loss of veins (e.g. *veinlet* and *vein)*, excess veins (e.g. *plexus* and *net)* and thick veins (e.g. *Notch* and *Delta)*. Diaz-Benjumea and Garcia-Bellido deduced from extensive genetic and clonal analyses of such mutants that wing veins are specified in the third larval instar, the cell proliferation phase of wing development, and that genes involved in regulating cell proliferation are also involved in cell fate decisions. They also showed that loss of vein mutants are epistatic to excess vein mutants and proposed that veins are formed at the crests of waves of morphogens in the wing anlage with different sets of genes controlling the amplitude of the waves and the distance between crests (Diaz-Benjumea and Garcia-Bellido, 1990).

Some of the genetic predictions have recently been born out by molecular studies. The mutant *veinlet (ve)*, a loss of vein mutant, is a wing-specific allele of *rhomboid (rho)* renamed rho^{ve} (Bier et al., 1990; Sturtevant et al., 1993). *rho* encodes a localized transmembrane protein that is component of the *Drosophila* EGF receptor (EGF-R) signaling pathway. Like its homologs in other organisms, *Drosophila* EGF-R is a transmembrane tyrosine kinase associated with cell division and determination of cell fate (Baker and Rubin, 1992; Clifford and Schüpbach, 1989; Zak and Shilo, 1992). Because of its localized expression *rho* is believed to restrict spatially the activity of EGF-R (Ruohola-Baker et al., 1993; Sturtevant et al., 1993). Three aspects of *rho* expression in wing discs implicate *rho* as a key element in determining the distal elements of the longitudinal veins. (a) In third instar wings, *rho* is expressed in narrow bands corresponding to the positions of the longitudinal veins with loss of expression in rho^{ve} leading to loss of vein. (b) Ectopic *rho* expression is associated with ectopic vein formation (Sturtevant et al., 1993). (c) Excess of vein mutants (e.g. *px* and *net)* show a corresponding pattern of excess *rho* expression in the third instar (Sturtevant and Bier, unpublished data). Thus, *rho* is important in the specification of wing veins.

Another category of wing mutant includes those with defects in the execution of a specified cell fate. For example, *Drosophila* integrins, transmembrane heterodimers, are components of the basal junctions that hold the two wing surfaces together (Fristrom et al., 1993). Mutations in integrin genes (e.g. *inflated (if)*, an α chain mutation and *myospheroid (mys)*, a β chain mutation) are defective in dorsal-ventral adhesion of intervein cells and result in wing blisters (Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990). Thus, integrins are one of many effectors of intervein differentiation.

In this paper, we investigate the genetic and developmental properties of *blistered (bs)* (Lindsley and Zimm, 1992), a mutation that has received little attention in previous studies. In contrast to integrin mutants where the wings are blistered but the vein pattern remains relatively undisturbed, *bs* mutations lead to an excess of veins as well as blisters. We describe several new *bs* alleles and show that, with two notable exceptions, *bs* appears to function autonomously. The phenotypic analysis is facilitated by phalloidin and laminin staining of pupal wings where the extent of vein and intervein can be more readily analyzed than in adult wings. We have also examined wild-type and *bs* wings by conventional microscopy throughout metamorphosis to establish the onset and progression of *bs* defects. These phenotypic analyses suggest that *bs* is required for the initiation of intervein development at the onset of metamorphosis and for limiting the extent of veins.

Finally, we have investigated the genetic interactions of representative *bs* alleles with the *rho*, *if* and *mys* mutants described above. The observation that *rho* suppresses the vein defects of *bs* mutants, suggests a regulatory role for *bs* in vein formation. This is supported by the observation that the extent of *rho* expression in *bs* wings increases during metamorphosis. The domains of ectopic *rho* expression closely correspond to the regions of increased vein. We propose a model for wing development in which the positions of the major veins, determined in part by the distribution of *rho*, is established by the end of the third instar, but that the final extent of veins, determined in part by the *bs* product, is not established until the onset of vein/intervein differentiation during metamorphosis. At this time, cells that fail to differentiate as intervein can still enter the vein pathway. Thus, progressively more severe *bs* mutants have progressively wider veins until the entire wing is, in effect, a vein.

METHODS AND MATERIALS

Mutant stocks and crosses

All stocks were maintained on standard cornmeal and molasses medium at 25°C. The origin of the various *bs* mutants is indicated in results.

Genetic mapping

Potential *bs* alleles were mapped with respect to the marker *Irregular facets (If)* which lies 0.3 map units distal to *bs*. A chromosome bearing a potential *bs* allele (bs^n) and *If* was placed *in trans* to a wild-type second chromosome. Females of this genotype were crossed to bs^{10} *If* homozygotes. Progeny, heterozygous for *If* and displaying the *bs* phenotype (bs^n $+/bs^{10}$ *If*) or homozygous for *If* and bs^+ (bs^+ $If/bs^{10}If$) were scored as recombinants. 800-1000 flies were scored for each new *bs* allele and in each case the new allele mapped to 0.3 map units of *If* (Gotwals, 1992).

Lethal phase analysis

To determine the lethal phase of the lethal *bs* alleles, bs^n (where n refers to any lethal *bs* allele) flies were crossed to wild-type flies. Heterozygotes were then backcrossed either to bs^n/Cyo or $Df(2R)Px^4/CyO$ animals. The only lethal progeny were bs^n homozygotes or $bs^n/Df(2R)Px^4$. Typically, 100 embryos from this cross were picked and their development followed by counting first, second and third instar larvae, pupae, pharate adults and adults. The lethal phase was determined by observing when 25% of the animals in a given cross died.

Mosaic analyses

Chromosomes carrying extreme *bs* alleles (bs^{12}, bs^{13} and bs^{14}) were marked with *pwn* (a mutation that causes wing hairs to be small and barbed (Lindsley and Zimm, 1992) and balanced over *CyO*. Somatic recombination was induced by X-irradiation (1,000 rads over 10 minutes) at 36, 60 and 84 (+/− 12) hours after egg laying. Wings of eclosed flies were mounted in euparol. 45 *pwn* clones in 1200 wings were identified and examined.

Whole mounts of pupal wings

Pupae were staged from pupariation (white prepupa) for 18 to 30 hours at 25°C. Staged pupae were immersed in 4% formaldehyde in PBS. A mid-dorsal incision was made with fine iridectomy scissors through both pupal case and body wall from the edge of the operculum to the posterior tip of the animal to admit fixative. Fixation was continued at room temperature on a shaker for at least one hour and

the fixed animals stored for up to one week at 4°C. Dissection of the wings was accomplished by removing the pupal case, grasping the wing hinge with fine forceps and gently pulling off the wing.

Dissected discs and wings were stained with FITC-phalloidin (Molecular Probes) or anti-laminin A (Henchcliffe et al., 1993) (kindly provided by Claire Hencliffe and Corey Goodman) as previously described (Fristrom et al., 1993). Stained wings were mounted in 50 to 75% glycerol in PBS containing 0.15% *p*-phenylenendiamine and examined by conventional immunofluorescence microscopy and by confocal microscopy (BIORAD 6000).

In situ hybribization of rhomboid probes used digoxigenin labelled RNA probes (Boehringer-Mannheim, 1093 657) as described previously (Sturtevant et al., 1993).

Light and electron microscopy

Larval and prepupal discs were dissected directly into fixative (1.5% glutaraldehyde, 0.5% formaldehyde in 0.1M sodium cacodylate). Pupal wings were dissected from animals fixed as described above then processed by standard procedures. 1µm sections for light microscopy were stained with toluidine blue and thin sections for electron microscopy were stained with uranyl acetate and lead citrate.

RESULTS

Genetic characterization of *blistered* mutants

The *blistered* locus (*bs*; 2-107.3) is uncovered by *Df(2R)Px2* and *Df(2R)Px4* which define the cytological region 60C5-6; 60D1. *bs* has previously been represented by relatively weak viable alleles (Gotwals and Fristrom, 1991; Lindsley and Zimm, 1992). We have identified a new set of *bs* alleles including some with extreme wing phenotypes and others that are lethal as homozygotes. A set of EMS induced mutations uncovered by both *Df(2R)Px2* and *Df(2R)Px4* (hereafter Px^4) were kindly provided by Elizabeth Raff and Robert Dettman. Five of these failed to complement viable *bs* alleles and mapped 0.3 map units centromere-proximal to *If*, coincident with the location of the *blistered* locus. We conclude that these five mutations are all *bs* alleles. Another allele p^{LFO6} was recovered in association with a nearby P-element insertion. Below, we rename and characterize each new *bs* allele in ascending order of severity (Table 1) – all are phenotypically more severe than existing alleles (Lindsley and Zimm, 1992). Two of the alleles are lethal but the basis for the lethality is not yet understood. bs^{14} behaves as a genetic null in that bs^{14} homozygotes and bs^{14}/*Df(2R)Px2* are both lethal as second to third instar larvae. Adult defects exhibited by the viable alleles appear to be restricted to the wings. Wing phenotypes range from ectopic venation and a moderate frequency of localized blisters (Fig. 1B) through very thick posterior veins and a high frequency of blisters (Fig. 1C) to a complete loss of adhesion between the two wing surfaces resulting in ballooned wings (Fig. 1D). Ballooned wings are typically composed entirely of small vein-like cells except for a strip in the center of the wing, corresponding to the intervein region between L3 and L4. This region is also resistant to ectopic vein formation in the weaker *bs* alleles and in other excess vein mutants (Diaz-Benjumea and Garcia-Bellido, 1990). In all the new *bs* mutants the wings are held out at an angle to the body axis and the animals are

Table 1. Genetic characterization of *blistered* mutants

Allele*	Previous designation	Lethal phase	Comment
bs^{10}	bs^{A48}	viable	blisters, ectopic veins
bs^{11}	*l(2)F61*	viable	blisters, wide veins and ectopic veins
bs^{12}	p^{LF06}	viable	blisters and balloons small cells
bs^{13}†	*l(2)F62*	pupal/pharate	
bs^{14}†	*l(2)F70*	2nd/3rd instar larvae	genetically amorphic
$Df(2R)bs^{F31}$‡	*l(2)F31*	embryonic	small deficiency

*Alleles are listed in order of severity.

†We have yet to establish unequivocally that the lethality associated with bs^{13} or bs^{14} is due to the blistered mutation. It is conceivable that these mutations are small deletions that also affect an adjacent gene required for viability. Both of these alleles, however, do not complement *bs* alleles, but fully complement all the known complementation groups uncovered by *Df(2R)PX2* and *Df(2R)PX4* (Kimble et al., 1990)

‡Southern blot analysis and chromosome in situ analysis demonstrate that $Df(2R)bs^{F31}$ removes more than 90 kilobases of DNA including the *Drosophila* muscarinic acetylcholine receptor gene (P. J. Gotwals and J. W. Fristrom, unpublished).

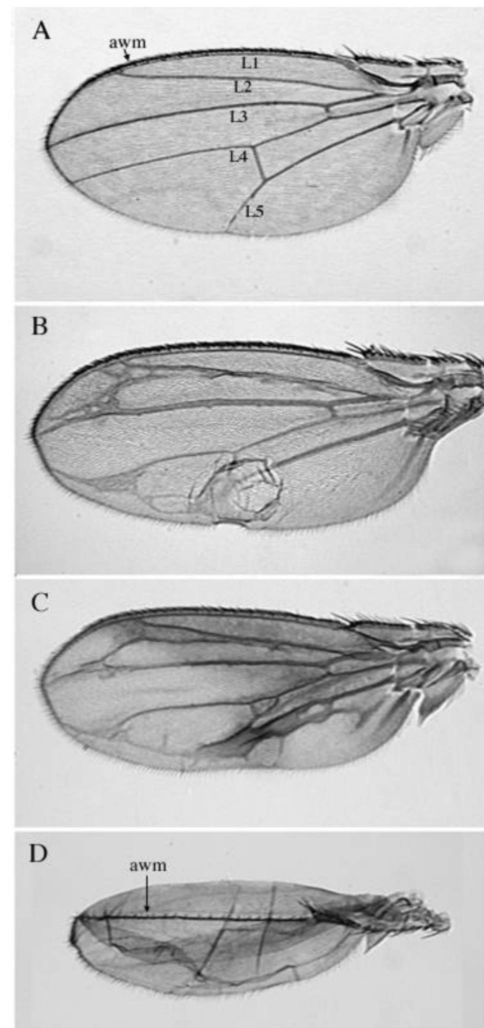


Fig. 1. Phenotypes of *bs* wings. (A) Wild type. (B) bs^{10} shows the stereotypical pattern of ectopic and thickened veins and posterior blister (C) bs^{11} shows ectopic veins as well as a very wide vein 5. Most bs^{11} wings also have large blisters (not shown). (D) bs^{12} shows a fully ballooned wing. *awm*, anterior wing margin; *L1-L5*, longitudinal veins 1 to 5.

flightless. However, even in the most severely defective wings, neural tissues such as the bristles associated with the anterior margin and the campaniform sensillae appear to develop normally.

Mosaic analysis

Mosaic analyses were undertaken to examine the wing phenotypes of the lethal alleles as well as to determine whether *bs* functions autonomously or non-autonamously. Homozygous mosaic patches of the lethal alleles bs^{13} and bs^{14} and the viable allele bs^{12} marked with the hair mutation *pwn* were generated. We observed both autonomous and non-autonomous aspects of the *bs* phenotype in mosaics.

Autonomous effects

Clones of the lethal allele bs^{14} were clearly autonomous with respect to the effects of *bs* on cell size. Homozygous bs^{14}/bs^{14} clones of all sizes in all regions of the wing contained cells that are considerably smaller, as judged by hair density, than either the adjacent cells or the overlying cells (Fig. 2A,B). Clones were usually yellowish in color and the lateral spacing of clone hairs closer than the proximal-distal spacing. Where clones cross a vein, the boundary between vein and intervein is obscure because all the clone cells resemble vein cells. Nevertheless, the center of the vein could usually be identified by two rows of hairs directed towards each other. All homozygous bs^{14} patches, including those between L3 and L4 are characterized by small vein-sized cells. In contrast, in wings of the most severe viable alleles, a strip of large intervein-sized cells persists in the region between L3 and L4. Thus, bs^{14} has a more severe wing phenotype than any of the known viable alleles. This, combined with its genetic behavior (above), makes bs^{14} a likely candidate for a null allele.

In contrast to bs^{14}, clones of the lethal bs^{13} showed a weak wing phenotype. Homozygous *pwn* patches only exhibited defects when they occurred in the vicinity of L5 and the anterior cross vein, a region that is particularly susceptible to ectopic vein formation. In this location, a bs^{13} clone typically contains an ectopic vein but not all the clone cells are necessarily vein-like. Thus, the wing phenotype of bs^{13} is comparable to that of the weak alleles bs^{2} or bs^{10}.

Non-autonomous effects

(a) Widening of dorsal veins overlying ventral clones. Veins L3 and L5 are referred to as dorsal veins because they exhibit dorsal bulges (L2 and part of L4 bulge ventrally (Garcia-Bellido, 1977)). We found several examples in all three of the alleles tested where ventral clones incorporating L3 resulted in thickening of the dorsal aspect of the vein from 4 to about 8-10 cells in width (Fig. 2C-E). A similar phenomenon was seen with ventral clones underlying L5. This may represent true non-autonomy of *bs*, i.e. expression of *bs* in ventral cells is required to inhibit vein formation in the overlying dorsal cells. Alternatively, there may be a general requirement for dorsal-ventral adhesion between cells immediately adjacent to the vein in order to prevent them from becoming vein-like.

(b) Blisters extend beyond clone borders. Virtually all *bs/bs* clones were associated with blisters that typically extended well beyond the boundaries of the clone. This could be a non-specific effect resulting from hydrostatic pressure within the blister mechanically disrupting dorsal/ventral connections in adjacent heterozygous tissue. A similar phenomenon is seen in wing clones of integrin mutants (Brower and Jaffe, 1989; Zusman et al., 1990). As transmembrane adhesion molecules integrins presumably function autonomously. However, in the case of *bs* mosaics where the nature of the product is unknown and blisters can occupy up to six times the area of the marked clone, the possibility of a true non-autonomous effect on intervein adhesion cannot be ruled out.

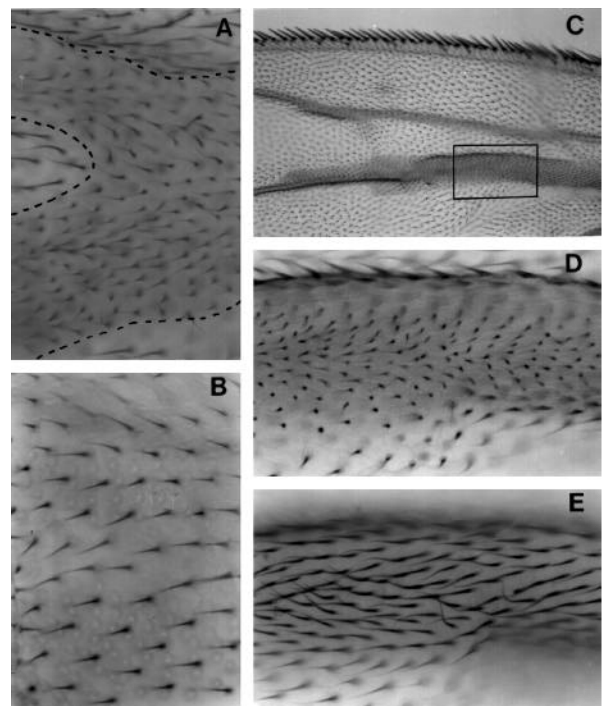


Fig. 2. Homozygous clones of bs^{14} marked with *pwn* (small hairs). (A,B) Autonomous effect of *bs* on cell size. (A) A dorsal clone (outlined) in the intervein region between L3 and L4. (B) Corresponding ventral surface. Note the high hair density within the clone compared with adjacent cells (A) and underlying cells (B). (C-E) Non-autonomous effect of *bs* on vein width. (C) Low magnification of a wing containing a clone associated with L3. (D,E) High magnifications of the boxed area in C. (D) Ventral surface containing clone. (E) Dorsal surface showing increased width of L3.

Developmental analysis

Pupal development

We have examined the morphogenesis of wild-type and *bs* wings during pupal development to characterize further the *bs* phenotype and to describe the normal sequence of events in vein/intervein differentiation. Phenotypic analysis of adult wings is limited by the impermeable cuticle, the death of intervein cells and the tendency for blistered wings to crumple. We have therefore examined pupal wings at 30 hours apf (after puparium formation), a stage when the vein pattern is complete and the wing is free of cuticle. Confocal microscopy of phalloidin-stained pupal wings was used to assess apical cell size and the extent of basal apposition. (Extensive actin filaments revealed by phalloidin-staining are located apically in association with the zonula adherens and in basal networks (Condic et al., 1990)). Laminin-staining was used to determine the extent of vein tissue (the acquisition of a laminin-containing

basal lamina appears to be a specific property of vein cells (Fristrom et al., 1993; Murray et al., personal communication)). We also used these techniques to examine the course of vein/intervein differentiation between 18 and 30 hours apf in both *bs* and wild-type wings. These studies provide some new insights into the normal process of vein/intervein differentiation as well as some clues to the basis of the blistered phenotype.

Wild type (Figs 3A-F; 4A-B)

Between 12 and 18 hours apf, the wild-type wing is a hollow sac composed of rapidly dividing unspecialized epithelial cells. The dorsal and ventral surfaces become reapposed as the basal surfaces of intervein cells extend processes to the opposite side (Fristrom et al., 1993; Waddington, 1941). Reapposition begins at 18 hours apf as longitudinal bands of cells, two to three cells in width, extend long processes basally to connect with their partners on the opposite surface (Fig. 3A). These 'intervein bands (IBs)' arise progressively from anterior to posterior in each of the five intervein regions so that by 21 hours apf, five IBs are present. Between 21 and 30 hours apf, the cells on either side of the IBs and adjacent to the wing margin reappose, progressively 'zipping' the intervein epithelia together until only the veins remain unapposed (Fig. 3C,E).

As intervein cells become apposed basally, vein cells become reduced in diameter apically. In the 18 hour wing, the final round of cytokinesis is taking place (Schubiger and Palka, 1987) with numerous large premitotic cells interspersed with smaller postmitotic cells (Fig. 3B). The average diameter of the postmitotic cells is 4.5 microns. By 21 hrs, broad bands of cells of smaller apical diameter (2.5 microns) appear (Fig. 3D). When apical and basal sections are superimposed it is evident that the small cells lie between the IBs, centered over broad basal channels (Fig. 3C,D). The bands of cells with small apical diameter subsequently become narrower and more sharply defined until 30 hours apf, when the veins have reached their final dimensions (of 2-4 rows of cells in width; Fig. 3E,F). Note that the decrease in apical diameter of vein cells is accompanied by an increase in intensity of phalloidin staining suggesting that the apical constriction is mediated by contraction of the circumapical actin filaments (Condic et al., 1990).

When reapposition of intervein cells is complete, the remaining unapposed channels form the vein lacunae. These channels become lined with laminin (Fristrom et al., 1993; Murray et al., personal communication), as detected by staining with an antibody to the *Drosophila* laminin A chain (Henchcliffe et al., 1993). Thus, the 30 hour apf laminin-stained wing resembles a miniature adult wing (Fig. 4A) with five longitudinal and two cross veins outlined by laminin staining (Fig. 4B). One difference between the pupal and adult wing is that a marginal channel extends around the entire periphery of the wing in pupae (and prepupae) but in adults there is no vein associated with the posterior wing margin. In pupae, not only is the entire marginal channel laminin stained, but the vein markers *rho* (Sturtevant et al., 1993) and *star* (Heberlein et al., 1993) are also expressed in this location, indicating that the posterior channel can be considered a transient vein.

In summary, intervein differentiation in wild-type wings involves the extension of basal processes that connect with cells on the opposite surface. This process begins in narrow intervein bands and progresses laterally until only the vein channels remain unapposed. Vein cells constrict apically and deposit a laminin-containing matrix basally.

blistered (Figs 3G-H; 4C-F)

At 30-36 hours apf there is a progressive increase in the extent of laminin-lined channels in progressively more severe *bs* mutants (Fig. 4C-F). We also find ectopic laminin-lined channels in areas that form ectopic veins in the adult (Fig. 4C,D). In bs^{12} wings, channels associated with L4 and L5 are often fused into a single large posterior 'vein' (Fig. 4E). In bs^{11}/Px^{4} the entire wing is a laminin-lined tube, except for a narrow band along the posterior border (Fig. 4F).

There was no evidence of intervein blisters in pupal wings; those regions that were not laminin-lined were basally apposed. Even wings of bs^{12}, which are typically completely ballooned in the adult, are always apposed to some extent in the anterior half of the wing and in a narrow band along the posterior margin (Fig. 4E). We conclude that blisters arise later, perhaps during the expansion stage, 50-60 hours apf (Fristrom et al., 1993) or during eclosion.

In phalloidin-stained pupal wings of *bs* mutants, we found, as expected, that cells of small apical diameter were associated with basal channels. Surprisingly, the distribution of cells with small apical diameter extends well beyond the corresponding basal channels. This is demonstrated in the bs^{11} wing shown in Fig. 3G,H. In basal view, the distal vein lacunae are only slightly wider than normal whereas in apical view small cells occupy the entire anterior and posterior thirds of the wing (as they often do in the adult wing). Thus, large areas of cells have properties of both vein and intervein: an apical diameter characteristic of vein cells and basal behavior characteristic of intervein cells. Cells of small diameter could arise in a number of ways; for example, defects in expansion during the preceding prepupal period could lead to abnormally small intervein cells. We therefore compared cell diameters in *bs* and wild-type wings at 18 hours apf when all postmitotic cells have a diameter characteristic of intervein cells. At this stage, wings of bs^{11} and bs^{12} were similar to wild type in both cell size (4.5 μm) and wing area (not shown). We tentatively conclude that wing cells of small apical diameter in at least some *bs* mutants arise by apical contraction during vein differentiation.

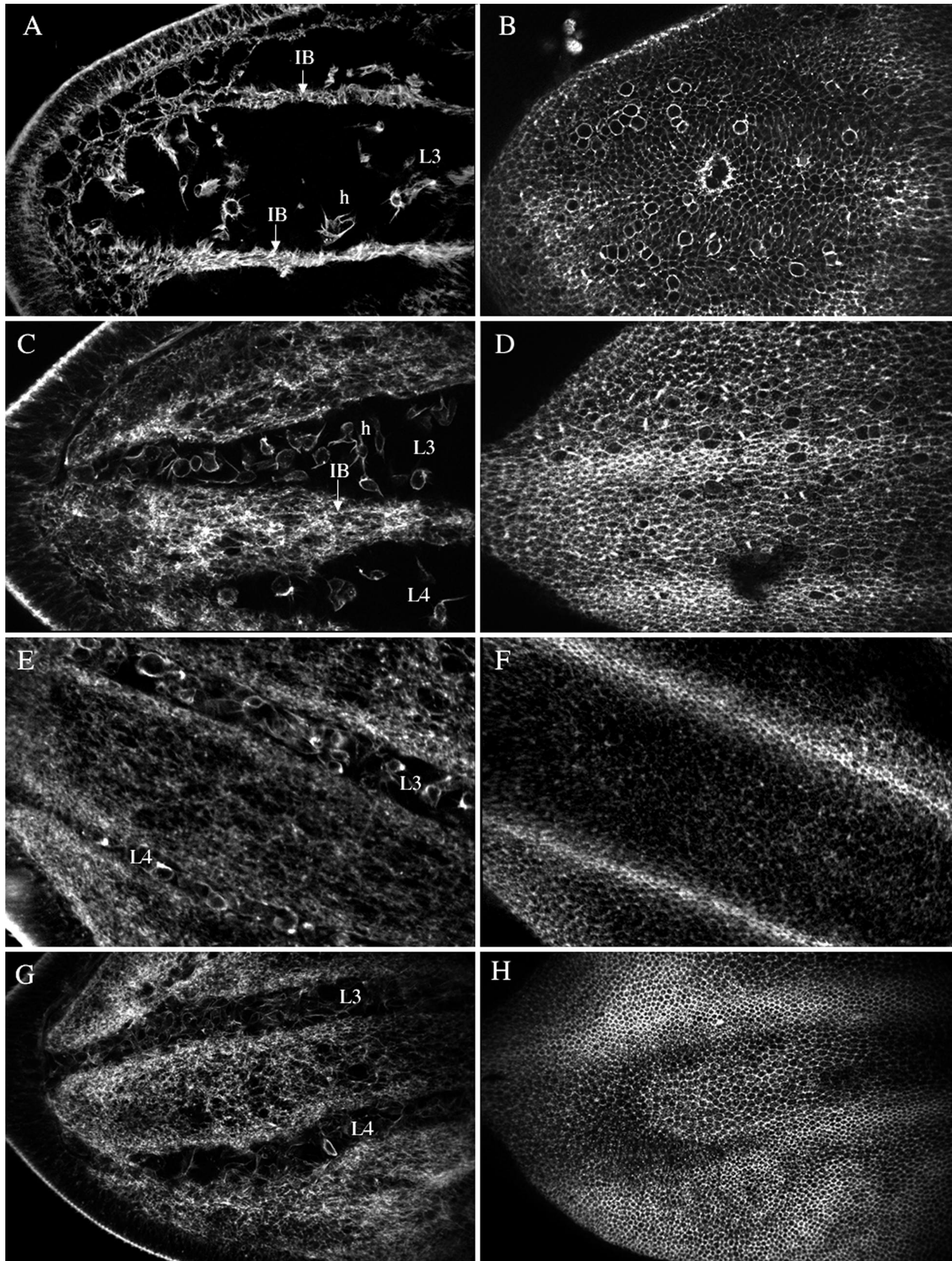
Prepupal development

To identify the earliest detectable defects in *bs* wings, we compared prepupal wings from the most extreme viable *bs* combination *(bs^{11}/Px^{4})* with those of wild type. The prepupal period, 0-12 hours apf, is the first stage of metamorphosis when the imaginal discs undergo morphogenesis to form the approximate shapes of the adult appendages. The wing disc, for example, is converted from a concentrically folded disc to a bilayered epithelium. Clear phenotypic defects in *bs* wings during the first few hours of the prepupal period presage equivalent defects seen during pupal development described above.

Wild type

Formation of the wing bilayer is accomplished largely by local changes in cell shape and size so that the wing disc folds along the wing margin bringing dorsal and ventral surfaces into apposition (Fristrom and Fristrom, 1993). The basal lamina of the

larval disc forms a potential barrier to the formation of cell-cell contacts between the apposed epithelia (Fig. 5A). Two different mechanisms appear to be involved in eliminating the larval basal lamina. (i) Beginning at the end of the third instar, cells in the vicinity of the wing margin detach from the basal lamina so that, as the wing margin expands and folds, the basal



lamina is left behind. This detachment may be facilitated by the secretion of a proteoglycan-containing network between the cells and the basal lamina (Brower et al., 1987). Cells on either side of the margin elongate until their basal cell surfaces make direct contact (Fig. 5A). (ii) In more central regions of the wing, the basal lamina becomes trapped between the apposed surfaces and is subsequently degraded so that, by 6 hours apf, it is no longer detectable as a discrete structure. The apposed basal surfaces make contact and differentiate the junctions of the prepupal transalar apparatus (Fristrom et al., 1993). Throughout the process of dorsoventral apposition the basal cell surfaces are highly convoluted and vesicular suggestive of secretory and/or endocytotic activity. Laminin-lined channels or preveins lie in the approximate positions of the future veins L3-L5 (Waddington, 1941; Murray, personal communication) but are broader and less clearly demarcated than pupal wing veins. No clear differences in apical cell diameter between prevein and intervein cells are evident. As in pupal wings, a narrow laminin-lined channel encompasses the entire wing margin.

blistered

Defects in wing discs of bs^{11}/Px^{4} become apparent in the first 2 hours after puparium formation. The distal and anterior submarginal cells detach from the larval basal lamina but do not become closely apposed resulting in an empty space between basal surface and basal lamina (Fig. 5B). In the central region, after the basal lamina disappears, the apposed basal surfaces are often in close proximity but show no tendency to make contact and form junctions. Ultrastructurally, the basal surfaces remain smooth and unvesiculated throughout the prepupal period (not shown). From 6 hours apf until the pupal period, a

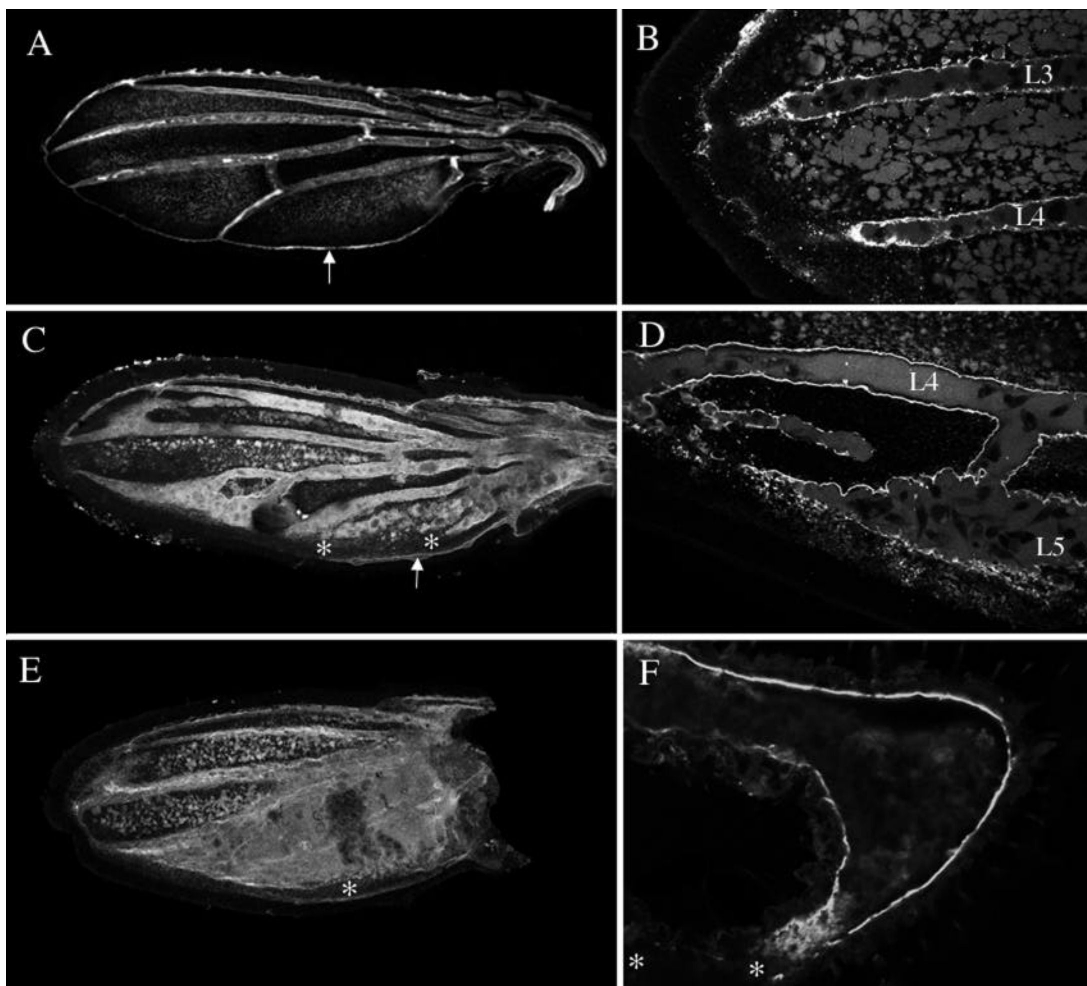


Fig. 4. Confocal micrographs of laminin-stained pupal wings. (A,B) Wild type; (C,D) bs^{11}; (E) bs^{12}; (F) bs^{11}/Px^{4}. (A-E) Whole mounts at 30 hours apf. (F) Transverse frozen section at 72 hours apf. Laminin staining shows the increased extent of vein channels in *bs* mutants (C,E) compared with wild type (A). Higher resolution images (B,D and F) reveal that laminin (intense signal) is concentrated at the periphery of the vein channels in both wild-type and *bs*. Areas of weak to moderate signal represent non-specific antibody binding to the wing extracellular matrix (Murray, personal communication). Hemocytes appear as unstained cells embedded in the vein matrix. L3-L5, longitudinal veins 3-5; arrow, posterior marginal vein; *, narrow posterior band that differentiates as intervein in even the most severe bs mutants (in sections adjacent to that shown in F, the region between * and * stained for integrin and microtubules confirming the intervein character of this region).

Fig. 3. Confocal micrographs of phalloidin-stained pupal wings from wild type (A-F) and bs^{11} (G-H). (A,B) 18 hours apf; (C,D) 21 hours apf; (E,F) 30 hours apf; (G,H) 30 hours apf. Left-hand panels show optical sections through the middle of the wing: reticulate staining identifies areas where intervein cells are basally apposed. Right-hand panels show corresponding apical sections, approximately 20 μm up: staining of junction-associated actin outlines individual cells. The bands of small, presumptive vein cells in D and F are centered over the basal channels in C and E. In bs^{11} wings (G,H), small cells in H extend far beyond the corresponding vein channels in G. IB, intervein band; L3-L4, the positions of the longitudinal veins 3 and 4; h, hemocytes. Distal is to the left.

single, large, hemocyte-filled, central channel stretches from the anterior margin and includes most of the wing except for a narrow band of apposed cell along the posterior margin comparable to the pupal wing shown in Fig. 4F. Prepupal discs from bs^{11} homozygotes, are intermediate between bs^{11}/Px^4 and wild type with abnormally wide, laminin-lined, prevein lacunae alternating with narrow stretches where dorsal and ventral surfaces have adhered. Thus, *bs* appears to have similar prepupal and pupal phenotypes; failure in apposition of intervein cells and expansion of vein territories.

Genetic interactions

To investigate *bs* function further, we examined the interaction of various *bs* alleles with mutations in genes whose specific role in wing development is understood.

blistered interactions with *rhomboid*

rhomboid (rho) is expressed where longitudinal veins will differentiate and is a component of the signaling pathway that specifies vein cell fate (see Introduction). rho^{ve} is a wing-specific allele of *rho* that results in lack of *rho* expression in wings along with the loss of the distal sections of longitudinal veins (Fig. 6A). We have constructed stocks homozygous for both rho^{ve} and the *bs* alleles bs^{10}, bs^{11} and bs^{12}. In general, rho^{ve} suppresses the vein defects associated with *bs* mutations but L2 to L5 remain truncated distally (Fig. 6B-D). *rho* is thus epistatic to *bs* with respect to vein formation. In combination with bs^{10} (Fig. 6B), rho^{ve} completely suppresses the formation of ectopic veins and blisters. Except for their 'held out' orientation, these wings cannot easily be distinguished from homozygous rho^{ve} alone. In combinations of rho^{ve} with the more severe *bs* alleles, veins remain wider than normal in the proximal part of the wing (Fig. 6D), an area beyond the domain of *rho* function.

The expression of *rho* in *bs* mutants *(bs^2, bs^{10} and bs^{11})* was examined. In late third instar discs, *rho* expression is restricted to narrow stripes corresponding to longitudinal veins in both *bs* and wild type (Fig. 7A,D). In very young prepupal wings (2 hours apf; Fig. 7B,E), the bands of *rho* expression associated with L2 and L5 in *bs* are wider and more intense than in wild type, a difference that becomes more pronounced by the tongue stage (4 hours apf; Fig. 7C,F). The expression of *rho* in pupal wings (30 hours apf) is also expanded (not shown). In bs^{11} there are broad bands of solid *rho* expression similar to the prepupal pattern, whereas in the weaker mutants bs^{10} and bs^2 there is a reticulate pattern of ectopic staining (Sturtevant and Bier, unpublished data). The increased area of *rho* expression thus anticipates the final vein phenotype of *bs* mutants. These observations lend strong support to the morphological evidence that the excess 'veins' of *bs* mutants represent true veins rather than a failure of intervein to differentiate. They also suggest that *bs* has a role in restricting the expression of *rho* during metamorphosis.

The effects of *bs* on *rho* expression do not account for all aspects of the *bs* phenotype and suggest that *bs* mutants have primary defects in intervein specification and/or differentiation. For example, rho^{ve} only weakly suppresses blisters. With the more severe alleles, bs^{11} and bs^{12}, a high frequency of blisters remains (about 50% with bs^{11} and 95% with bs^{12}). Indeed, in combination with bs^{12}, the wings often remain balloon-like. So even though, in the absence of *rho*, veins are suppressed, the remaining intervein regions often fail to adhere dorsoventrally. We note that rho^{ve} does effectively (100% of the progeny) suppress the posterior blisters associated with bs^{10}. This might be an indirect result of eliminating ectopic veins in this region of the wing i.e. increased area of adhesion may compensate for a presumed decrease in strength of adhesion between the two wing surfaces. rho^{ve} also fails effectively to suppress the non-vein-associated decrease in cell size (see Fig. 3H) and consequently the wings remain small (Fig. 6C,D).

blistered interactions with mutations in integrin subunits

A role for *bs* in intervein formation is further supported by genetic interactions between *bs* and integrin mutants. Integrins, transmembrane adhesion proteins, are expressed only in the intervein regions of pupal wings where they eventually become localized to intercellular junctions between the dorsal and ventral wing surface (Fristrom et al., 1993). Viable mutations of *if* (α2 integrin) and somatic clones of lethal *mys* (β integrin)

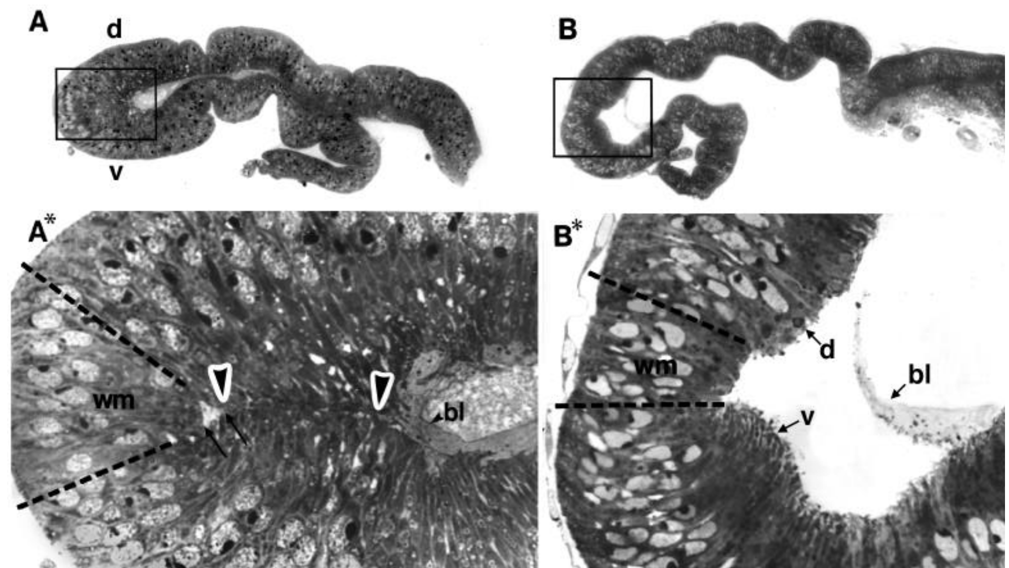


Fig. 5. Longitudinal 1 μm sections through prepupal wings of wild-type and *bs* wings at 2 hours apf. (A,A*) Wild-type. The wing disc has folded along the wing margin (wm) and cells on either side of the margin have become apposed (between arrowheads) except for a small triangular gap, the marginal vein (small arrows). The basal lamina (bl) has detached from the region of apposed cells but elsewhere persists between dorsal and ventral epithelia. (B,B*) bs^{11}/Px^4. The basal lamina (bl) has detached from the cells adjacent to the margin but dorsal (d) and ventral (v) surfaces have not adhered. Note that in both wild type and *bs*, cells of the wing margin (between dotted lines) are shorter than cells on either side.

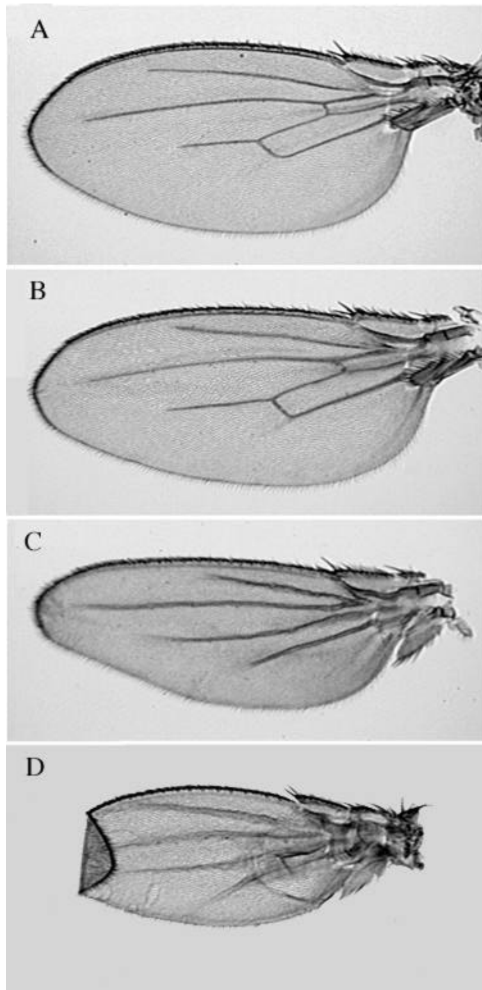


Fig. 6. Wings from flies homozygous for rho^{ve} and *bs* alleles. (A) bs^{+}; (B) bs^{10}; (C) bs^{11}; (D) bs^{12}. In all cases, the rho^{ve} phenotype of distally truncated veins is evident indicating that *rho* is epistatic to *bs*. Vein defects of the *bs* mutants are suppressed (cf. Fig. 1). Intervein defects such as small cell size leading to reduced wing size, persist (C,D) and a high frequency of blistered and ballooned wings occur in bs^{11} and bs^{12} homozygotes (not shown but see text).

mutations result in adult wing blisters similar to those seen in weak *bs* mutations (Brower and Jaffe, 1989; Zusman et al., 1990) but cause little or no disruption in the normal vein pattern. We looked for dominant interactions between integrin and *bs* mutations by crossing females carrying *if* (if^{3}) or *mys* (mys^{nj}) to *bs* males. There was an increase in frequency and severity of blisters in most of the combinations examined particularly with the more severe *bs* alleles. (see also Wessendorf et al., 1992). For example, $if^{3}/Y;\ bs^{defF31}/+$ results in 100% of wings having blisters (compared with 24% in if^{3}/Y; +/+). For the most part blistered/integrin combinations did not affect venation even when the blisters were very large.

DISCUSSION

The metamorphosis of the larval wing disc into an adult wing takes place in two stages: the prepupal period (0-12 hours apf) and the pupal period (12-96 hours apf). In the prepupal period, the dorsal and ventral wing epithelia become apposed, converting the concentrically folded wing disc to a bilayered wing. A coarse pattern of unapposed ‘prevein channels’ is established, presaging the events of the pupal period. At the onset of the pupal period, the wing layers separate, revert to an unspecialized state and undergo further cell division. Definitive wing differentiation begins around 18 hours apf with the progressive reapposition of dorsal and ventral epithelia (Fig. 3). Reapposition begins at the center of intervein regions and proceeds laterally until, by 30 hours apf, only the vein channels remain unapposed. After reapposition of intervein regions has commenced, future vein cells constrict in apical area. When intervein reapposition is complete, vein cells become coated basally by a laminin-containing ECM and laminin is cleared from the intervein regions.

We have identified a series of mutants that fail to complement each other and existing *bs* mutants and that map to the *bs* locus 0.3 map units proximal to *If*. These have therefore been designated as *bs* alleles and numbered bs^{10}-bs^{14} in increasing order of severity. Of these new alleles, three are viable and two are lethal as homozygotes. Viable alleles and mosaic patches of lethal alleles are phenotypically characterized by defects in wing development.

Increasingly severe *bs* mutants differentiate vein over increasingly large areas of the wing as judged by the extent of laminin-lined channels and the expansion of *rho* expression in prepupal and pupal wings as well as by the adult phenotype. The suppression of extra vein in *bs* mutants by rho^{ve} (a mutant lacking *rho* expression in wings) further supports the view that the extra vein in *bs* wings represent true veins. We conclude that loss of bs^{+} function results in the conversion of intervein to vein. In addition to extra vein, *bs* mutants show intervein defects such as loss of adhesion between dorsal and ventral surfaces leading to the blisters from which the mutant is named. Rho^{ve} only weakly suppresses such defects. In contrast, mutations in integrin genes (effectors of intervein adhesion during pupal development) interact dominantly with *bs* mutants to increase the frequency of intervein blisters. Taken together, the results indicate that *bs* functions both in intervein formation and in determining extent of vein. We suggest that these two processes are closely linked during development and propose the following model.

Vein/intervein differentiation

It is well established that the positions of the longitudinal veins are defined by the end of the third larval instar. We propose (1) that the lateral extent of veins remains labile until the pupal period and (2) that the progress of intervein specification and/or differentiation during metamorphosis limits the extent of vein formation. Characteristics of mutations in *bs* (described here) and *Notch (N),* a transmembrane receptor involved in cell fate determination (Kidd et al., 1989) support this view as follows.

(1) Modifications of vein width occur late in development

(a) In severe *bs* mutants much of the wing is converted to vein but *rho* expression in such mutants is not affected until early in the prepupal period. Thus, the excess veins exhibited by *bs* mutants presumably arise after the onset of metamorphosis. This is consistent with the observation that *rho* functions in

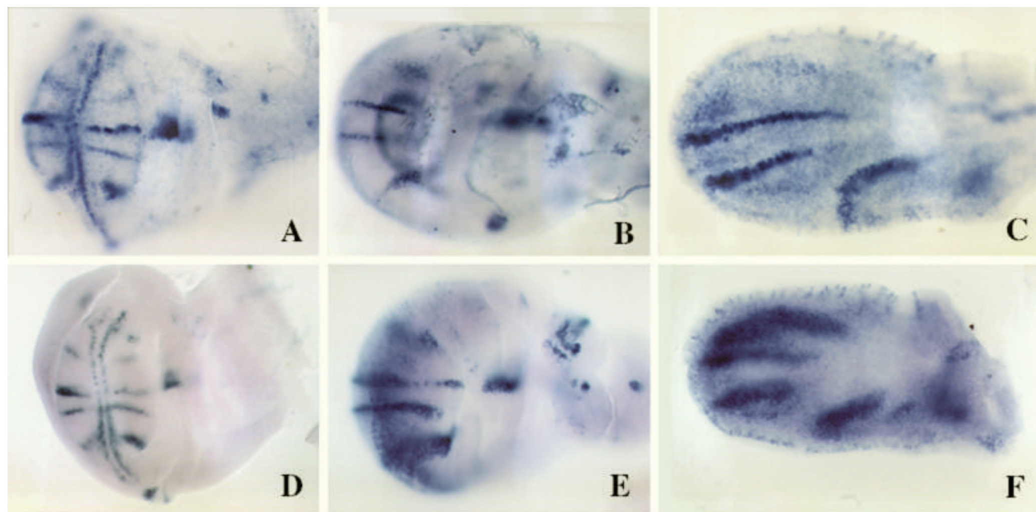


Fig. 7. *Rho* expression in wild-type (A-C) and bs^{11} (D-F) wing anlagen at different stages of development. (A,D) Late third instar; (B,E) 2 hours apf; (C,F) 4 hours apf. Note the expanded areas of *rho* expression in *bs* wings at 2 and 4 hours apf.

vein formation throughout most of metamorphosis (Sturtevant and Bier, unpublished data). We conclude that one function of *bs* is directly or indirectly to delimit the prepupal and pupal expression of *rho* and thereby prevent the acquisition of extra vein during metamorphosis.

(b) Recent work with *N* also demonstrates that vein width can be modified as late as 24 hours apf. One of many phenotypes associated with *N*, is a slight increase in vein thickness. This defect occurs during the pupal period as demonstrated by the temperature-sensitive period of N^{ts} (Shellenbarger and Mohler, 1978) and for *N* deletion constructs missing all or part of the extracellular domain (Rebay et al., 1993).

(2) Intervein development affects vein width

(a) Three lines of evidence indicate that *bs* functions in intervein cells. First, morphological defects in severe *bs* mutants are evident by 2 hours apf suggesting that *bs* has a primary defect in intervein formation, which *precedes* its effect on *rho* expression. Second, intervein defects persist in severe *bs* mutants even when excess veins are suppressed by rho^{ve}. Third, *bs* appears autonomously to convert intervein to vein in all regions of the wing. It is therefore likely that *bs* is expressed throughout intervein regions at least by the onset of metamorphosis. (It is also possible that *bs* has an earlier function (in larval discs) that is not manifest until morphogenesis begins.)

(b) *N* is expressed predominantly in intervein regions (Fehon et al., 1991) and is presumably involved in signalling formation of intervein at the expense of vein (Heitzler and Simpson, 1991). The slight increase in vein width associated with loss of *N* function is strongly dominantly enhanced by *bs* (L. Wessendorf and D. Fristrom, unpublished data). Although it is premature to conclude that *bs* is part of a *N* signalling pathway, both these genes are evidently expressed in intervein cells and affect vein width.

The morphogenetic events involved in the pupal differentiation of intervein and vein (summarized in Fig. 8) are consistent with this model. Intervein differentiation begins in longitudinal stripes midway between the future veins and expands laterally until the veins are reached. At about the same time, stripes of smaller vein cells become evident. The filling in of the regions between the alternating stripes of vein and intervein

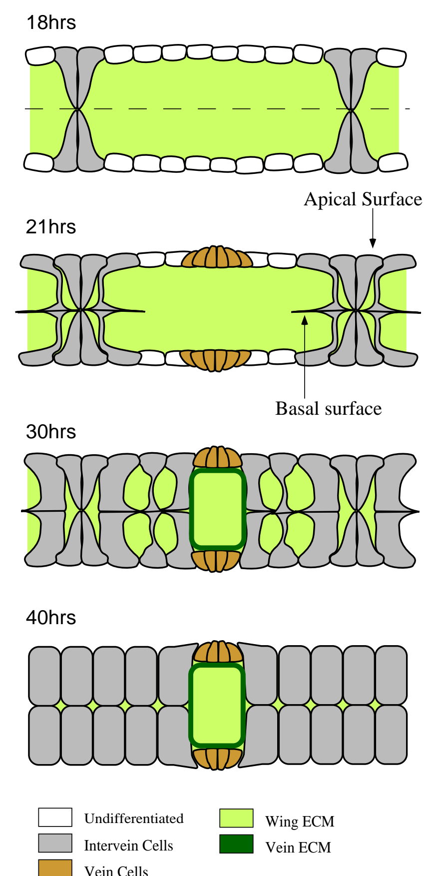


Fig. 8. A schematic summary of the initial phases of vein and intervein differentiation. At around 18 hours apf, pairs of intervein cells (grey) on opposite surfaces become connected by basal extensions to form an intervein band. At around 21 hours apf, vein cells (brown) characterized by small apical diameter arise between intervein bands. Between 21 and 30 hours apf, reapposition of intervein cells progresses laterally from the intervein band until all but the vein cells are basally apposed. Finally, the extracellular spaces in the intervein regions disappear.

may involve a complex two-way lateral signalling system that establishes cell identity as morphogenesis proceeds.

In the context of this model, we view the *bs* product as

necessary for the triggering or initiation of intervein formation, first manifested by the apposition of dorsal and ventral epithelia. When bs^+ function is limiting, intervein differentiation fails to expand laterally or in the most severe cases fails to begin; *rho* expression expands and vein forms in place of intervein. We cannot yet predict the nature of the *bs* product; possibilities range from a signalling molecule required for intervein identity to a transcription factor required for regulating downstream components of the intervein pathway or even an effector molecule (e.g. a basal lamina degrading protease) required early in intervein differentiation. The cloning and characterization of the *bs* gene will undoubtedly contribute to a better understanding of the processes involved in cell fate decisions during wing development.

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REFERENCES

Baker, N. and Rubin, G. M. (1992). Ellipse mutations in the *Drosophila* homolog of the EGF receptor affect pattern formation, cell division and cell death in eye imaginal discs. *Dev. Biol.* **150,** 381-396.

Bier, E., Jan, L. Y. and Jan, Y. N. (1990). *Rhomboid*, a gene required for dorso-ventral axis establishment and peripheral nervous system development in *Drosophila melanogaster. Genes Dev.* **4,** 190-203.

Blair, S. S. (1994). A role for the segment polarity gene *shaggy-zeste white 3* in the specification of regional identity in the developing wing of *Drosophila. Dev. Biol.* **162,** 229-244.

Brower, D. L. and Jaffe, S. M. (1989). Requirement for integrins during *Drosophila* wing development. *Nature* **342,** 285-287.

Brower, D. L., Piovant, M., Salatino, R., Brailey, J. and Hendrix, M. J. C. (1987). Identification of a specialized extracellular matrix component in *Drosophila* imaginal discs. *Dev. Biol.* **119,** 373-381.

Clifford, R. J. and Schüpbach, T. (1989). Coordinately and differentially mutable activities of torpedo, the *Drosophila melanogaster* homolog of the vertebrate EGF receptor gene. *Genetics* **123,** 771-787.

Condic, M. L., Fristrom, D. and Fristrom, J. W. (1990). Apical cell shape changes during *Drosophila* imaginal leg disc elongation: a novel morphogenetic mechanism. *Development* **111,** 23-33.

Couso, J. P., Bishop, S. A. and Martinez Arias, A. (1994). The *wingless* signalling pathway and the patterning of the wing margin in *Drosophila.. Development* **120,** 621-636.

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

Diaz-Benjumea, F. J. and Hafen, E. (1994). The *sevenless* signalling cassette mediates *Drosophila* EGF receptor function during epidermal development. *Development* **120,** 569-578.

Dobzhansky, T. (1929). The influence of quantity and quality of chromosomal material on the size of cells in *Drosophila melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.* **115,** 363-379.

Fehon, G. R., Johansen, K., Rebay, I. and Artavanis-Tsakonas, S. (1991). Complex cellular and subcellular regulation of *Notch* expression during embryonic and imaginal development of *Drosophila*: Implications for *Notch* function. *J. Cell Biol.* **113**, 657-669.

Fristrom, D. and Fristrom, J. (1993). The metamorphic development of the adult epidermis. *In 'The Development of Drosophila'* (ed. A. Martinas-Arias and M. Bates), pp. 843-897. Cold Spring Harbor: Cold Spring Harbor Press.

Fristrom, D., Wilcox, M. and Fristrom, J. W. (1993). The distribution of PS integrins, Laminin A and F-actin during key stages in *Drosophila* wing development. *Development* **117,** 509-523.

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

Garcia-Bellido, A. and De Celis, J. F. (1992). Developmental genetics of the venation pattern of *Drosophila*. *Ann. Rev. Genet.* **26,** 277-304.

Gotwals, P. J. (1992). Genetic interactions identify genes involved in *Drosophila* imaginal disc morphogenesis. Ph.D. Thesis University of California, Berkeley.

Gotwals, P. J. and Fristrom, J. W. (1991). Three neighboring genes interact with the Broad-complex and the Stubble-stubbloid locus to affect imaginal disc morphogenesis in *Drosophila*. *Genetics* **127,** 747-759.

Heberlein, U., Hariharan, I. K. and Rubin, G. M. (1993). *Star* is required for neuronal differentiation in the *Drosophila* retina and displays dosage-sensitive interactions with *Ras 1. Dev. Biol.* **160,** 51-63.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64,** 1083-1092.

Henchcliffe, C., Garcia-Alonso, L., Tang, J. and Goodman, C. S. (1993). Genetic analysis of Laminin A reveals diverse functions during morphogenesis in *Drosophila*. *Development* **118,** 325-337.

Johnson, S. A. and Milner, M. J. (1987). The final stages of wing development in *Drosophila melanogaster. Tissue Cell* **19,** 505-513.

Kidd, S., Baylies, M. K., Gasic, G. P. and Young, M. W. (1989). Structure and distribution of the Notch protein in developing *Drosophila*. *Genes Dev.* **3,** 1113-1129.

Kimble, M., Dettman, R. W. and Raff, E. C. (1990) The β3-tubulin gene of *Drosophila melanogaster* is essential for viability and fertility. *Genetics* **126**, 991-1005.

Lindsley, D. L. and Zimm, G. G. (1992). The genome of *Drosophila melanogaster*. New York: Academic Press. Inc.

Rebay, L., Fehon, R. G. and Artavanis-Tsakonas, S. (1993). Specific truncations of *Drosophila Notch* define dominant activated and dominant negative forms of the receptor. *Cell* **74,** 319-329.

Ruohola-Baker, H., Grell, E., Chou, T.-B., Baker, D., Jan, L. Y. and Jan, Y. N. (1993). Spatially localized *rhomboid* is required for establishment of the dorsal-ventral axis in *Drosophila* oogenesis. *Cell* **73,** 953-965.

Schubiger, M. and Palka, J. (1987). Changing spatial patterns of DNA replication in the developing wing of *Drosophila*. *Dev. Biol.* **123,** 145-153.

Shellenbarger, D. L. and Mohler, J. D. (1978). Temperature-sensitive periods and autonomy of pleiotropic effects of $l(1)N^{ts1}$, a conditional *Notch* lethal in *Drosophila. Dev. Biol.* **62**, 432-446.

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

Tucker, J. B., Milner, M. J., Currie, D. A., Muir, J. W., Forrest, D. A. and Spencer, M. (1986). Centrosomal microtubule-organizing centres and a switch in the control of protofilament number for cell surface-associated microtubules during *Drosophila* wing morphogenesis. *Eur. J. Cell Biol.* **41,** 279-289.

Waddington, C. H. (1941). The genetic control of wing development in *Drosophila*. *J. Genet.* **41,** 75-139.

Wessendorf, L. H. V., Wehrli, M., DiAntonio, A. and Wilcox, M. (1992). Genetic interactions with integrins during wing morphogenesis in *Drosophila.. Cold Spring Harbor Symp. Quant. Biol.* **57**, 241-248.

Wilcox, M., DiAntonio, A. and Leptin, M. (1989). The function of PS integrins in *Drosophila* wing morphogenesis. *Development* **107,** 891-897.

Williams, J. A., Paddock, S. W., and Carroll, S. B. (1993) Pattern formation in a secondary field: A heirarchy of regulatory genes subdivides the developing *Drosophila* wing disc into discrete sub-regions. *Development* **117,** 571-584.

Zak, N. B. and Shilo, B. (1992). Localization of DER and the pattern of cell divisions in wild type and *Ellipse* eye imaginal discs. *Dev. Biol.* **149,** 448-456.

Zusman, S., Patel-King, R. S., French-constant, C. and Hynes, R. O. (1990). Requirements for integrins during *Drosophila* development. *Development* **108,** 391-402.

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