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Activation of the *knirps* locus links patterning to morphogenesis of the second wing vein in *Drosophila*

Karen Lunde[1,2], Jennifer L. Trimble[1], Annabel Guichard[1], Kirsten A. Guss[3], Ulrich Nauber[4] and Ethan Bier[1,*]

[1]Section of Cell and Developmental Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0349, USA
[2]Albert-Ludwigs-Universität Freiburg, Institut für Biologie I, Hauptstrasse 1, D-79104 Freiburg, Germany
[3]Department of Biology, Dickinson College, PO Box 1773, Carlisle, PA 17013, USA
[4]Max-Planck-Institut für biophysikalische Chemie, Abt. Molekulare Entwicklungsbiologie, Am Fassberg 11, D-37077 Göttingen, Germany
*Author for correspondence (e-mail: bier@biomail.ucsd.edu)

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SUMMARY

The adjacent *knirps* (*kni*) and *knirps-related* (*knrl*) genes encode functionally related zinc finger transcription factors that collaborate to initiate development of the second longitudinal wing vein (L2). *kni* and *knrl* are expressed in the third instar larval wing disc in a narrow stripe of cells just anterior to the broad central zone of cells expressing high levels of the related *spalt* genes. Here, we identify a 1.4 kb *cis*-acting enhancer element from the *kni* locus that faithfully directs gene expression in the L2 primordium. We find that three independent *ri* alleles have alterations mapping within the L2-enhancer element and show that two of these observed lesions eliminate the ability of the enhancer element to direct gene expression in the L2 primordium. The L2 enhancer can be subdivided into distinct activation and repression domains. The activation domain mediates the combined action of the general wing activator Scalloped and a putative locally provided factor, the activity of which is abrogated by a single nucleotide alteration in the *ri*^{53j} mutant. We also find that misexpression of genes in L2 that are normally expressed in veins other than L2 results in abnormal L2 development. These experiments provide a mechanistic basis for understanding how *kni* and *knrl* link AP patterning to morphogenesis of the L2 vein by orchestrating the expression of a selective subset of vein-promoting genes in the L2 primordium.

Key words: *knirps*, *radius incompletus*, L2 vein, wing, *Drosophila melanogaster*, Patterning, Morphogenesis

INTRODUCTION

One of the important remaining questions in developmental biology is to understand how positional information is linked to the genesis of specific morphological structures in different locations of the organism. The *Drosophila* wing is a system that is particularly well suited for studying the relationship between patterning and morphogenesis. For example, mechanisms for establishing positional information along the anterior-posterior (AP) axis have been analyzed in detail (reviewed by Lawrence and Struhl, 1996; Strigini and Cohen, 1999; Klein, 2001) and morphological structures such as the five major longitudinal veins (L1-L5) form at invariant positions along the AP axis of the adult wing (reviewed by García-Bellido and de Celis, 1992; Bier, 2000). As described below, the five wing veins can be distinguished from one another by several criteria including: expression of specific combinations of genes during development; whether they are located primarily on the dorsal or ventral surfaces of the wing (odd versus even numbered veins respectively); and by the types or absence of sensory organs that form along them (e.g. sensory organs form on the L1 and L3 veins, but not on the L2, L4 or L5 veins). An interesting question then, is how vein-specific as well as more general vein developmental programs are initiated at various locations in response to different positional information.

Wing vein development in *Drosophila* can be subdivided into two temporally distinct stages: initiation and differentiation. Vein initiation begins during the mid-third larval instar (Waddington, 1940; García-Bellido, 1977; García-Bellido and de Celis, 1992; Sturtevant et al., 1993; Sturtevant and Bier, 1995; Bier, 2000) when the wing imaginal disc is a monolayer of cells. The wing blade proper derives from an oval region of the wing disc known as the wing pouch, while the remainder of the disc generates elements of the wing hinge and thoracic body wall. Vein differentiation, the second phase of vein development, occurs during metamorphosis as the wing disc buds out (or everts), folding along the future wing margin. Ultimately, this eversion leads to the apposition of the dorsal and ventral surfaces of the wing during pupal development, creating the bilayer of cells comprising the mature wing blade.

Genes involved in initiating wing vein development (vein genes) are expressed during the third larval instar in narrow stripes, corresponding to vein primordia, or in broader ‘pro-

vein' stripes, consisting of cells that are competent to become vein cells (Biehs et al., 1998). Some vein genes are expressed in all vein primordia, while others are expressed in subsets of veins or in single veins. For example *rhomboid* (*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 Egfr signaling pathway (Sturtevant et al., 1993; Noll et al., 1994; Bier, 1998a; Bier, 1998b; Guichard et al., 1999). *caupolican* (*caup*) and the neighboring gene *araucan* (*ara*) encode related homeobox genes that promote expression of other vein genes such as *rho* in odd numbered veins. Proneural genes such as *achaete* (*ac*) and *scute* (*sc*) promote neural development in the L1 and L3 primordium (Gomez-Skarmata et al., 1996). *Delta* (*Dl*) encodes a ligand for the Notch (N) receptor, which mediates lateral inhibitory interactions among cells in vein-competent domains during pupal development (Shellenbarger and Mohler, 1978; Kooh et al., 1993; Parody and Muskavitch, 1993). *Delta* is also expressed earlier during larval stages of wing development in all longitudinal veins except L2, and is likely to play a role in limiting vein thickness at this stage as well, since loss of Notch function during larval development leads to greatly broadened expression of the vein marker *rho* (Sturtevant and Bier, 1995). *kni* and *knrl*, which encode related zinc finger transcription factors in the steroid hormone superfamily (Nauber et al., 1988; Oro et al., 1988; Rothe et al., 1989), are expressed in a single stripe corresponding to the L2 primordium beginning in the mid-third larval instar, and are required to initiate L2 development (Lunde et al., 1998). *kni* also functions as a gap gene in early embryonic development (Nüsslein-Volhard and Wieschaus, 1980; Nauber et al., 1988).

The L2 stripe of *kni/knrl*-expressing cells forms along the anterior border of a broad domain of cells expressing high levels of the related and functionally overlapping *spalt-major* (*salm*) (Kühnlein et al., 1994) and *spalt-related* (*salr*) (Barrio et al., 1996) zinc finger transcription factors. A variety of evidence indicates that central domain cells expressing the patterning genes *salm* and *salr* (together referred to as *sal*) induce their anterior neighbors, which express very low levels of *sal*, to become the L2 primordium (Sturtevant et al., 1997; Lunde et al., 1998). For example, in wings containing *salm*-mutant clones, ectopic branches of L2 are induced that track along and inside the $salm^{-}$ clone borders, mimicking the normal situation in which an L2 vein forms just outside the domain of high-level *sal*-expressing cells (Sturtevant et al., 1997). This ability of *sal*-expressing cells to induce their anterior low-level *sal*-expressing neighbors to initiate L2 development requires the activity of the *kni* locus (Lunde et al., 1998). The induction of *kni*/*knrl* expression in the L2 primordium therefore provides an excellent system for studying the transition from spatial patterning to tissue morphogenesis.

Once activated along the anterior *sal* border, *kni* and *knrl* organize development of the L2 vein, in part by activating expression of the key vein-promoting gene *rho* and by suppressing expression of the intervein gene *blistered* (*bs*) (Montagne et al., 1996; Lunde et al., 1998). The *kni* locus is highly selective in regulating downstream gene expression in the L2 primordium as revealed by the observation that several genes expressed in other veins, such as *caup* and *ara*, *ac* and *sc*, and *Delta*, are excluded from the L2 primordium. Thus, the *kni* locus links patterning to vein-specific morphogenesis by functioning downstream of *sal* and upstream of genes involved in vein versus intervein development.

The role of the *kni* locus in L2 formation has been clarified by analysis of likely regulatory alleles of the *kni* locus, previously known as *radius incompletus* (*ri*) (Arajärvi and Hannah-Alava, 1969; Lunde et al., 1998). Flies with this mutation lack large sections of the L2 vein. kni^{ri} mutants are homozygous viable, in contrast to *kni* null mutants, which die as embryos with a gap gene phenotype (Nüsslein-Volhard and Wieschaus, 1980; Nauber et al., 1988). In support of kni^{ri} mutations being wing-specific regulatory alleles of the *kni/knrl* locus, expression of *kni* and *knrl* in the L2 primordium is absent in $kni^{ri[1]}$ mutants, and the L2 vein-loss phenotype can be partially rescued by ubiquitous expression of *kni* in the wing (Lunde et al., 1998).

The findings summarized above suggest that *kni* and *knrl* organize the L2 vein developmental program by orchestrating expression of genes that execute distinct subsets of functions required for proper L2 development. Several important unanswered questions remain, including: what function(s) are disrupted in $kni^{ri[1]}$ and other existing kni^{ri} mutants?; do these mutations eliminate the function of an L2-specific *cis*-regulatory element in the *kni* locus?; and finally, with respect to the definition of L2 versus other veins, is it important to exclude expression of genes expressed in veins other than the L2 primordium?

In this study we report the identification of an enhancer element upstream of the *kni* coding region that selectively directs gene expression in the L2 primordium in third instar larval wing discs. We show that three separate *ri* alleles have defects mapping within a minimal 1.4 kb L2 enhancer element. We demonstrate that two of these mutations eliminate activity of the L2 enhancer, $kni^{ri[1]}$, which contains a 252 bp deletion, and $kni^{ri[53j]}$, which harbors a single base-pair substitution. We find that truncation of the minimal L2 enhancer to a 0.69 kb fragment leads to ectopic reporter gene expression in the extreme anterior and posterior regions of the wing, indicating that repression contributes to restricting activation of the L2 enhancer. In addition, we show that the general wing promoting transcription factor Scalloped (Sd) binds with high affinity to several sites in the L2 enhancer and that *sd* is required for *kni* expression in the wing disc. We have also employed the L2 enhancer element as a tool to drive expression of various UAS transgenes in the L2 primordium. We find that the loss of the L2 vein in *ri* mutants can be rescued by L2-specific expression of either the *kni* or *knrl* genes, or the downstream target gene *rho*. In addition, we find that misexpression of genes in the L2 primordium that are normally expressed in veins other than L2 results in abnormal L2 development. These results provide a framework for understanding how positional information is converted into morphogenesis of the L2 wing vein by 'vein organizing genes' such as *kni* and *knrl*.

MATERIALS AND METHODS

Fly stocks

The weak $kni^{ri[M3]}$ allele isolated by J. Díaz-Benjumea was kindly

provided by Antonio Gárcia-Bellido (Universidad Autonoma de Madrid); Ruth Lehmann (Skirball Institute, New York) provided the strong viable *Df(3L)kni*$^{ri[XT2]}$ allele and the Df(3L)*kni*^{FC82} stock (Lehmann, 1985); Peter Portin (University of Turku) provided the *kni*$^{ri[92f]}$ allele; Yuh Nung Jan provided the *UAS-scute* stock (Chien et al., 1996); Juan Modolell (Universidad Autonoma De Madrid) provided the UAS-*ara* and UAS-*caup* stocks; Amanda Simcox (Ohio State University) provided the UAS-*vein* stock (Simcox et al., 1996); Joachim Urban (Universitat Mainz) provided the UAS-*eg* stock (Dittrich et al., 1997); Ken Irvine (Waksman Institute) provided the *w sd*^{58} P{*ry*$^{+}$, *hs-neo, FRT*} 18A stock, and Seth Blair (University of Wisconsin-Madison) provided the hsflp3MKRS/TM6B stock. *kni*$^{ri[M*]}$ and *kni*$^{ri[53j]}$ were obtained from the Bowling Green Stock Center, where they have since been abandoned. *kni*$^{ri[53j]}$ is a medium-strength homozygous viable allele isolated by Irwin Oster, a student of H. J. Müller, and *kni*$^{ri[M*]}$ is a medium-strength homozygous *ri* allele from the collection of H. J. Müller (Müller, 1941). Other mutant lines, including the medium-strength *kni*$^{ri[1]}$ allele, balancers and chromosomal markers were obtained from either the Bloomington Indiana Stock Center or the Tübingen Stock Center and are described by Lindsley and Grell (Lindsley and Grell, 1968) and Lindsley and Zimm (Lindsley and Zimm, 1992).

Vector construction

Genomic fragments spanning the majority of the putative regulatory region of the *kni* locus (Fig. 1) delimited by *Df(3L)kni*$^{ri[XT2]}$ (Nauber et al., 1988) were subcloned into the *pC4PLZ* expression vector (Wharton and Crews, 1993). *Eco*RI fragments F, E, S, R and Q (isolated from phages provided by U. Nauber) were inserted into pBluescript II KS *(+/–)* (*pBS*, Stratagene) with the *Not*I and T7 promoter containing the side of the vector most proximal to the *knirps* coding region. The constructs were subcloned into the *lacZ* transformation vector *pC4PLZ*. Subfragments of the 4.8 kb *Eco*RI fragment (fragment E in Fig. 1) that drive expression in the L2 primordium were then subcloned into *pC4PLZ* to define a minimal L2 enhancer element. To assay fragments of E, *pBS*-E was digested; the proximal most *Eco*RI-*Sal*I (ES, 2.0 kb), *Eco*RI-*Xho*I (EX, 1.4 kb), and *Eco*RI-*Hin*cII (EC, 0.69 kb) fragments isolated and re-inserted into *pBS*. The 4.8 kb fragment E was also subcloned into the *pC4PG4* expression vector, in which the *lacZ* gene in *pC4PLZ* has been replaced by *GAL4* (Emery, 1996), and was used to drive expression of various UAS transgenes in the L2 primordium.

Mapping of *kni* and *ri* breakpoints

Restriction fragments isolated from a lambda phage walk (Nauber et al., 1988) covering over 50 kb of the *kni* upstream region, delimited by the *Df(3L)kni*$^{ri[XT2]}$ deletion, were used as probes to determine the locations of potential chromosomal abnormalities such as deletions or rearrangements in *ri* mutants on Southern blots (Southern, 1975). Several different restriction enzymes were used to scan each region to distinguish single nucleotide polymorphisms from larger scale abnormalities such as those present in the *kni*$^{ri[1]}$ and *kni*$^{ri[M3]}$ mutants.

Isolation of *kni*^{ri} genomic fragments and construction of the E[*ri*]-*lacZ* vectors

Primers AA and RK were used to amplify a region surrounding the proximal *Eco*RI-*Bgl*II (1.197 kb) fragment of E from *kni*$^{ri[1]}$, *kni*$^{ri[92f]}$, *kni*$^{ri[M*]}$, and *kni*$^{ri[53j]}$ genomic DNA (AA=GACACAATGCTCCG-AATTCC, the 5′ end is in F, the 3′ end contains the *Eco*RI site; RK=CCCAATGGACCCCAATCTGGTTGGGG, the 5′ end is at 1.231 kb in E. Note that TGGGG was added to produce a *Kpn*I site at the distal end of the resulting fragment). PCR fragments were purified for sequencing using the QIAquick PCR Purification kit. The blunt ended *kni*$^{ri[1]}$ fragment was cloned into *pCR-BluntII-TOPO* (TOPO) and oriented so that the *Not*I site within TOPO and the *Bgl*II site within the fragment (*N*, *B*) are on opposite sides. The resulting *TOPO-N,B* construct and *pBS*-E were digested with *Not*I and *Bgl*II, fragments isolated and ligated to form *pBS-EΔri*[1]. The *EΔri*[1] insert was sequenced to confirm that it differed from the wild-type sequence by only the 252 bp deletion. *Kpn*I and *Not*I were used to remove *EΔri*[1], and it was cloned into the corresponding sites of *C4PLZ* to form *EΔri*[1]-*lacZ*. Similarly, primers ECO-*ri* and XBA-*ri* were used to PCR amplify the *Eco*RI-*Xho*I fragment of E (termed EX) from *kni*$^{ri[53j]}$ genomic DNA (ECO-*ri*=GAATTCCAACGCGAA-GCGTC; XBA-*ri*=TCTAGATGGGGCTGCTGCCA). The resulting 1.4 kb product was cloned into the TOPO vector. The EX(*ri*^{53j}) fragment was digested with *Eco*RI and *Xba*I and subcloned into the corresponding sites of *pC4PLZ* to form *EX*(*ri*^{53j})-*lacZ*. A single subclone was sequenced to verify that only the single nucleotide change (C596A) had been incorporated.

Generation and analysis of *sd*$^{-}$ clones

Generation of reduction-of-function *sd*^{58} clones and immunohistochemical analysis were performed as described previously (Guss et al., 2001).

Analysis of Sd binding sites in the L2 enhancer

Identification and analysis of Sd binding sites was performed as described by Guss et al. (Guss et al., 2001). Sequences of the upper strand of oligonucleotides used as probes for gel mobility shift assays and as PCR primers to introduce mutations into the Sd binding sites of the *kni* 0.69 kb element (fragment EC in Fig. 1F) are listed below in the 5′ to 3′ orientation. Altered bases are shown in lowercase in the mutant version, and the corresponding bases are underlined in the native sequence as follows:

kni 271 wild type: TTCCCCTCTTACATTTGTCGCATAGTTCC-CATCTTGGCCA

kni 271 mutant: TTCCCCTCTTtCATTaGgCtCATAaggCCCAT-CTTGGCCA

kni 570 wild type: TTGCGGACACAGGACACGAAATGCGTTT-TGTGCCTTAATT

kni 570 mutant: TTGCGGACACAGGACACctAATGaGgTTTGT-GCCTTAATT

kni 640 wild type: TTGCTGGTGCACTGAAAGAAATAGTTGA-AGGGATTATTG

kni 640 mutant: TTGCTGGTGCACTGAAcctAATAGgTGAAGG-GATTATTG

Reporter constructs with three mutated Sd binding sites were made by cloning PCR-generated fragments of fragment EC into the Hsp *lacZ*-CaSpeR plasmid (Nelson and Laughton, 1993). Four independent *EC-3Sdmut-lacZ* transformants were recovered and tested for *lacZ* expression.

Mounting fly wings

Wings from adult flies were dissected in isopropanol and mounted in 100% Canada Balsam mounting medium (Aldrich #28,292-8) as described previously (Roberts, 1986).

In situ hybridization to whole-mount larval wing discs

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

RESULTS

An L2 enhancer element lies upstream of the *knirps* coding region

In a previous study, we presented genetic evidence that the *kni*$^{ri[1]}$ mutation, as well as an approximately 50 kb deletion of *kni* upstream sequences (*Df(3L)kni*$^{ri[XT2]}$), disrupt the function of a wing-specific regulatory element in the *kni* locus that is

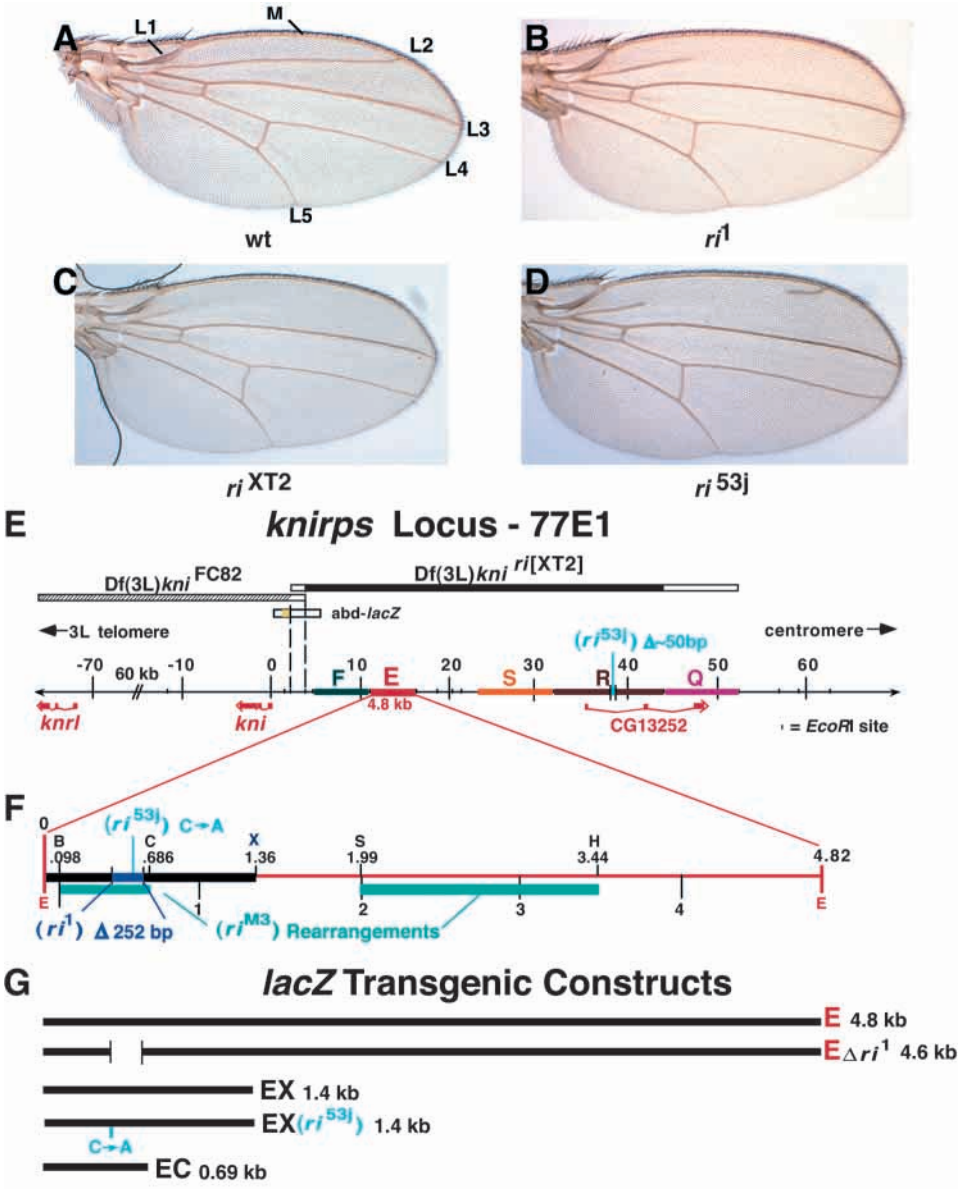

Fig. 1. Map of *knirps* locus and *ri* alleles. (A) A wild-type adult male wing. Longitudinal wing veins (L1-L5), and margin (M) are indicated. (B) A *kni*$^{ri[1]}$ homozygous adult male wing, with an incomplete L2 vein. Note that this phenotype is somewhat milder than that of *Df(3L)kni*$^{ri[XT2]}$ mutants (C). (C) A *Df(3L)kni*$^{ri[XT2]}$ homozygous adult male wing. Note the virtual absence of the L2 vein. (D) A *kni*$^{ri[53j]}$ homozygous adult male wing. (E) Diagram of region 77E1 in the left arm of the third chromosome showing *kni* and *knrl* transcripts, the regions deleted in *Df(3L)kni*$^{ri[XT2]}$ and *Df(3L)kni*^{FC82} as well as *lacZ* test constructs (*Eco*RI fragments) E (red), F (green), S (orange), R (brown), and Q (pink). The fragment labeled *abd-lacZ* (white box) has been shown to contain an embryonic enhancer (yellow box) driving expression in abdominal segments (Pankratz et al., 1992). This fragment does not drive gene expression in the wing primordium. Genomic DNA fragments between E and S were not tested in *lacZ* fusions. The 4.8 kb fragment E drives reporter gene expression in L2. abd (yellow box), embryonic abdominal enhancer (Pankratz et al., 1992); CG13252, a predicted protein-coding transcript that is expressed ubiquitously in both wild-type and *kni*$^{ri[53j]}$ wing discs (data not shown). The *knrl* transcript, which comprises 23 kb of genomic DNA (Rothe et al., 1992), is not drawn to scale. The 5′ end of the *knrl* gene is at –74.8 kb on this map. (F) Expanded map of 4.8 kb fragment E, showing the 1.4 kb minimal L2 driver (black), 252 bp *kni*$^{ri[1]}$ deletion (dark blue), C to A base substitution at 0.596 kb in E in *kni*$^{ri[53j]}$ (light blue), and the region of insertion(s) or rearrangements in *kni*$^{ri[M3]}$ (turquoise), from 0.098 to 0.686, and from 1.99 to 3.44 kb. E, *Eco*RI; B, *Bam*HI; C, *Hin*cII; X, *Xho*I; S, *Sal*I; H, *Hin*dIII. (G) *lacZ* transgenic constructs made to assay expression driven by a region containing the *kni*$^{ri[1]}$ deficiency substituted into E (*EΔri*[1]), and successively smaller 3′ subfragments of E: EX, 1.4 kb; and EC, 0.69 kb.

responsible for driving expression of the *kni* and *knrl* genes in the L2 primordium (Lunde et al., 1998). As a consequence of the loss of *kni*/*knrl* expression, *kni*$^{ri[1]}$ (Fig. 1B) and *Df(3L)kni*$^{ri[XT2]}$ (Fig. 1C) flies have severely truncated L2 veins (compare with the wild-type vein pattern in Fig. 1A). As a first step in extending these studies, we gathered and characterized four other putative independently generated viable *ri* alleles of the *kni* locus, which lack portions of the L2 vein, including *kni*$^{ri[53j]}$ (Fig. 1D), *kni*$^{ri[M3]}$, *kni*$^{ri[92f]}$, and *kni*$^{ri[M*]}$ (not shown). As in *kni*$^{ri[1]}$ wing discs, we found that *kni* expression is eliminated in the L2 primordium of each of these *kni*^{ri} alleles (data not shown).

The studies mentioned above suggested that there might be an L2-specific wing vein enhancer that controls expression of *kni* and *knrl* in the L2 primordium. We searched for such an enhancer element in two ways. First, we screened for the ability of genomic fragments upstream of the *kni* coding region to drive *lacZ* expression in the L2 primordium (summarized in Fig. 1E). Second, we used Southern blot analysis to identify deletions or rearrangement breakpoints in the genomic DNA of *ri* mutants in the *kni* upstream region uncovered by *Df(3L)kni*$^{ri[XT2]}$ (Fig. 1E, see below). As a result of the former effort, we identified a single 4.8 kb *Eco*RI fragment (fragment E in Fig. 1E-G) that drives *lacZ* reporter gene expression in a sharp stripe (Fig. 2B) similar to that of endogenous *kni* expression in the L2 primordium (Fig. 2A). Double label experiments confirm that *lacZ* expression driven by the *E-lacZ* construct coincides with that of endogenous *kni* expression (Fig. 2C). Additionally, the *E-lacZ* expression pattern includes a weaker stripe in the posterior region of the wing (Fig. 2B, arrow). A faint endogenous *kni* stripe can also be observed in this location in overstained preparations of wild-type discs (data not shown). Consistent with previous studies (Lunde et al., 1998), the *E-lacZ* L2 stripe is located just anterior to the broad domain of strong *salm*

expression (Fig. 2D). Notably, the weaker posterior *E-lacZ* stripe forms immediately adjacent to the posterior border of the *salm* domain (Fig. 2D).

In order to delimit a minimal L2 enhancer element, we tested the ability of 5′ truncated derivatives of the 4.8 kb fragment E (Fig. 1G) to drive reporter gene expression in larval wing imaginal discs. We observed that subfragments of 2.0 kb (*Eco*RI-*Sal*I, data not shown) and 1.4 kb (the *Eco*RI-*Xho*I fragment EX, Fig. 1G) drive expression of *lacZ* (Fig. 2E) in patterns nearly indistinguishable from that of the 4.8 kb *E-lacZ* construct (Fig. 2B). Further truncation generating a 0.69 kb *Eco*RI-*Hin*cII fragment (EC, Fig. 1G), however, results in high levels of ectopic *lacZ* expression in broad anterior and posterior domains (Fig. 2F). This observation indicates that regulatory element(s) required to repress gene expression in the extreme anterior and posterior domains of the wing primordium reside between the distal 0.69 kb and 1.4 kb of fragment EX (repression domain, Fig. 3B), and that sequences sufficient for activation are contained within the proximal 0.69 kb fragment EC (activation domain, Fig. 3A). As discussed below, we have identified sequences necessary for L2 activity in the activation domain as well as potential repressor binding sites in the proposed repression domain (Fig. 3A,B, Fig. 7).

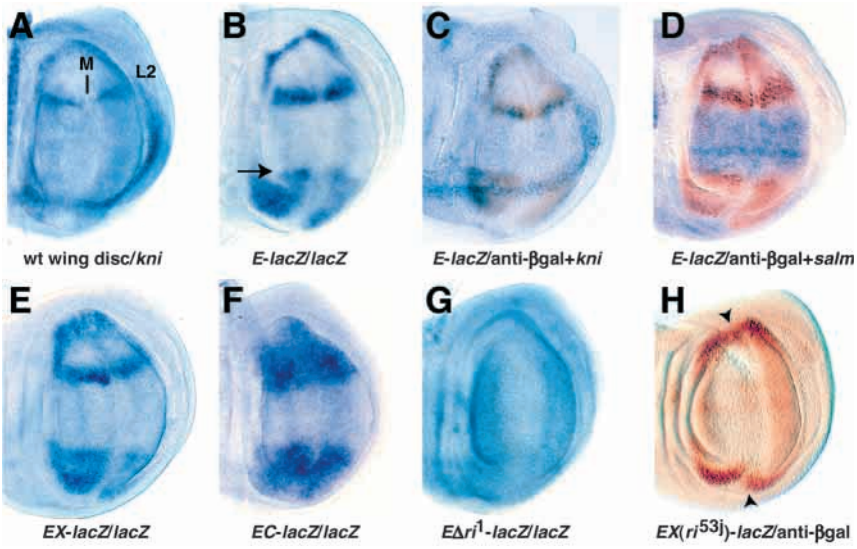

Fig. 2. Expression of wild-type and mutant L2 enhancer constructs. (A) *kni* expression in a wild-type mid-third instar larval wing disc. The L2 primordium (L2) and margin (M) are indicated. (B) *lacZ* expression driven by the *E-lacZ* construct in a mid-third instar larval wing disc. Note the lower levels of *lacZ* expression in a diffuse stripe in the posterior region of the wing pouch (arrow). (C) *E-lacZ* expression relative to endogenous *kni* expression in a mid-third instar larval wing disc double-stained for *kni* mRNA expression (blue) and anti-β-galactosidase protein (brown). Note the high degree of coincidence of these two expression patterns. (D) *E-lacZ* expression relative to *salm* expression in a mid-third instar larval wing disc double-stained for anti-β-galactosidase protein (brown) and *salm* expression (blue). Note the abutting and mutually exclusive expression of these two genes. (E) *EX-lacZ* expression driven by fragment EX (1.4 kb 3′ subfragment of E; see Fig. 1G) in a mid-third instar larval wing disc. (F) *EC-lacZ* expression driven by fragment EC (0.69 kb 3′ subfragment of E; see Fig. 1G) in a mid-third instar larval wing disc. Note the broad anterior and posterior domains of ectopic *lacZ* expression relative to that driven by the *E-lacZ* and *EX-lacZ* constructs. (G) *E-Δri*[1]*-lacZ* expression driven by the fragment *E-Δri*[1], which is deleted for the same 252 bp segment that is missing in the *ri*[1] allele, in a mid-third instar larval wing disc. Note the entire absence of patterned staining, including that observed in the posterior region of the wings discs (e.g. compare with staining in panel D). (H) *EX*(ri^{53j})*-lacZ* expression driven by the fragment EX(ri^{53j}), which contains the same single base pair alteration present in the $kni^{ri[53j]}$ allele, in a mid-third instar larval wing disc. Note that L2 staining is selectively lost while expression in extreme anterior and posterior domains of the wing pouch is retained (arrowheads: compare with staining in G).

ri mutants have lesions in the L2 enhancer that disrupt its function

As a complement to screening genomic fragments upstream of the *kni* locus for the ability to drive gene expression in the L2 primordium using *lacZ* promoter fusion constructs, we scanned the approximately 50 kb region deleted in the *Df(3L)kni*$^{ri[XT2]}$ allele for detectable aberrations in five putative independently isolated *ri* alleles. Using a series of probes spanning that complete genomic region (Fig. 1E), we found that a single *Eco*RI fragment (the same fragment E that drives expression in the L2 primordium) contains aberrations in the $kni^{ri[1]}$, $kni^{ri[92f]}$, $kni^{ri[M^*]}$ and $kni^{ri[M3]}$ mutants. For each of these alleles, mutations map within the minimal 1.4 kb L2 enhancer element (see Fig. 1F). Although no large-scale lesions were detected by Southern blot analysis within fragment E of the $kni^{ri[53j]}$ mutant, subsequent sequence analysis identified a single base pair alteration within the minimal L2 enhancer element EX (see below). The $kni^{ri[53j]}$ allele is also associated with a small deletion (approx. 50 bp) in fragment R.

Further molecular analysis of the various *ri* alleles revealed that the putative independently derived $kni^{ri[1]}$, $kni^{ri[92f]}$, and $kni^{ri[M^*]}$ alleles all contain the same 252 bp deletion. As this deletion is not flanked by duplicated sequences that could promote frequent identical recombination events, it may be that the $kni^{ri[92f]}$ and $kni^{ri[M^*]}$ alleles are actually re-isolates of the original $kni^{ri[1]}$ mutation. Consistent with these three alleles having a common origin, they also share a restriction enzyme polymorphism (i.e. loss of a *Hin*dIII site) in fragment Q (Fig. 1E) that is not shared by the other *ri* mutants or our *white*$^-$ control stock. To test whether the 252 bp deletion within the minimal L2 enhancer element is responsible for the loss of *kni* and *knrl* gene expression in $kni^{ri[1]}$ mutants, we made a $kni^{ri[1]}$-mutated *E-lacZ* construct. We amplified genomic DNA containing the $kni^{ri[1]}$ mutation and substituted it for the corresponding part of the original *E-lacZ* construct to generate E*Δri*^{1}*-lacZ* (Fig. 1G) and transformed this construct into flies. We examined *lacZ* expression in third instar larval wing discs of *EΔri*^{1}*-lacZ* flies and found that they failed to express *lacZ* in the L2 primordium or anywhere else in the wing pouch (Fig. 2G), indicating that the 252 bp deletion in $kni^{ri[1]}$ mutants eliminates the activity of the L2 enhancer element.

Since Southern blot analysis did not reveal any obvious lesions in fragment E of the $kni^{ri[53j]}$ allele, we considered the possibility that this allele might have a subtler lesion in the L2 enhancer region. Accordingly, we PCR amplified and

sequenced the 1.4 kb fragment (EX, Fig. 1G) corresponding to the minimal L2 enhancer from *kni*$^{ri[53j]}$. We found only a single nucleotide difference between the sequence of this mutant allele and the wild-type fragment EX (C596A, Fig. 3A), which lies within the same 252 bp region deleted in *kni*$^{ri[1]}$. Although it has been observed that single nucleotide mutations can eliminate endogenous enhancer function (Shimell et al., 1994), such cases are sufficiently rare that it was important, as in the

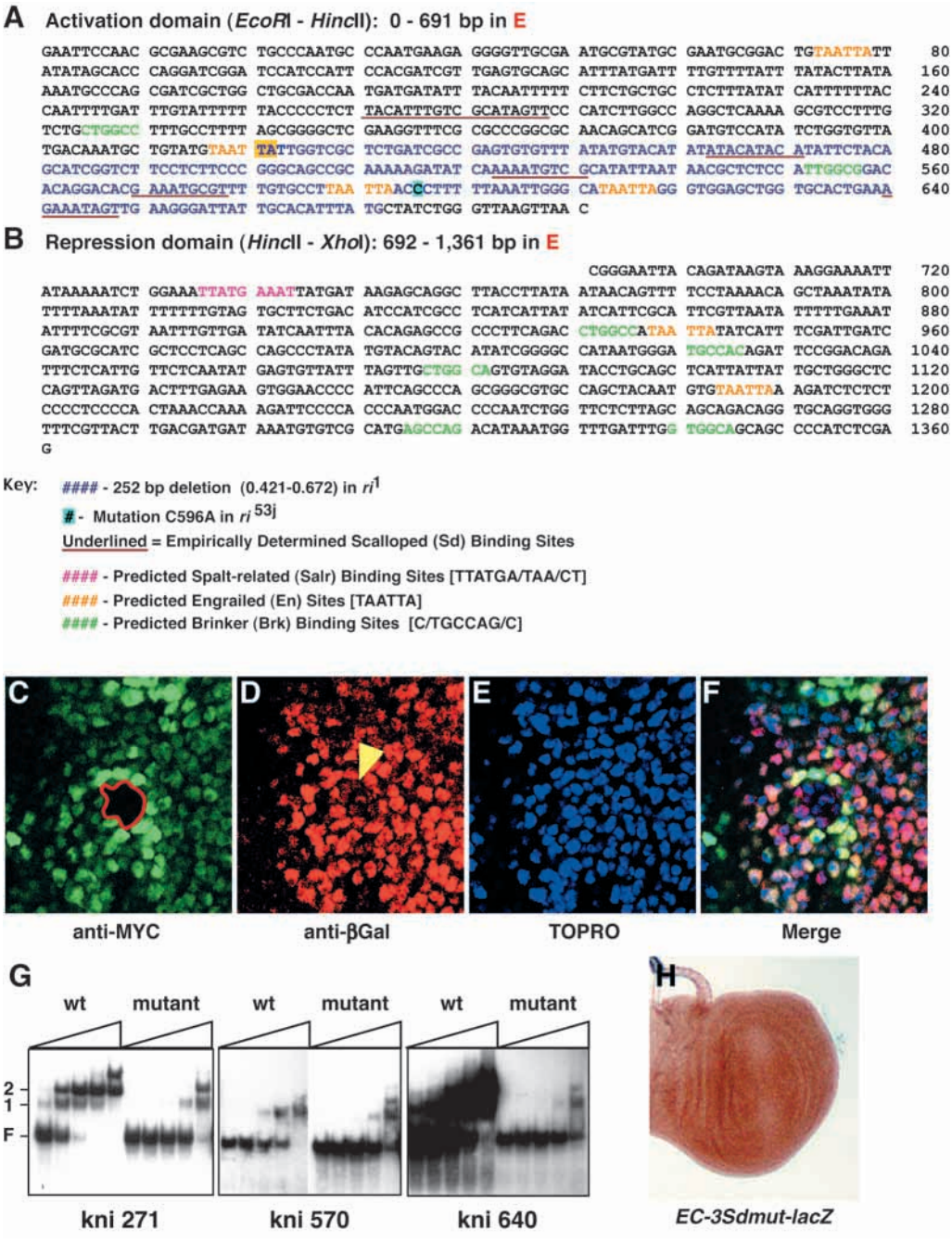

Fig. 3. The L2-enhancer element is activated by Sd. (A) Sequence of the *Eco*RI-*Hin*cII fragment (EC) of E, which contains sites necessary for activation of the minimal L2 enhancer (0 to 0.691 kb of fragment E). (B) Sequence of the *Hin*cII-*Xho*I fragment, which contains sequences required to repress ectopic activity of the L2 enhancer element (0.692 to 1.361 kb of fragment EX). Key: The deletion in the *kni*$^{ri[1]}$ mutant is indicated by blue text and the nucleotide mutated in the *kni*$^{ri[53j]}$ mutant (C to A at 596) highlighted in turquoise. Sd binding sites in fragment EC (underlined in brown) were determined empirically by footprinting and gel shifts. A tandem doublet of binding sites begins at 271 [TACATTTGTCGCATAGTT], while the sites beginning at 463 [TGTATGTAT] (opposite strand), 523 [AAAATGTCG], 570 [GAAATGCGT], and 640 [ACTATTTCT] (opposite strand) are single sites. These experimentally determined sites define a consensus [(A/T)(A/G)NAT(G/T)TNT], which matches well with the consensus [WRVWATKYR] derived for Sd binding to other wing enhancers that require *vestigial* and *sd* function such as *bs=DSRF* (Halder et al., 1998), *cut*, *sal* and the *vg* quadrant element (Guss et al., 2001). Other predicted DNA binding sites are indicated based on matches to known consensus sequences. Engrailed (En) [TAATTA – yellow type]: (Ades and Sauer, 1994); Spalt-related (Salr) [TTATGa/tAa/cT – pink type]: (Barrio et al., 1996); Brinker (Brk) [c/tGCCAg/c – green type]: (Rushlow et al., 2001; Zhang et al., 2001). No predicted DNA binding sites were identified in fragment EX for Mad (Kim et al., 1997), Ci (Kinzler and Vogelstein, 1990), or Kni (Small et al., 1996). (C-F) Reporter gene expression driven by the 4.8 kb element E is dependent upon *sd* function. (C) A mitotic clone homozygous for the hypomorphic *sd*^{58} allele located in the wing pouch is marked by the absence of the MYC epitope tag and is outlined in red. (D) Expression of the *E-lacZ* reporter gene is eliminated or reduced by the reduction of *sd* function, except in clones along the wing margin, where it is unaffected (data not shown). The yellow arrowhead indicates the position of the *sd*$^{-}$ clone. (E) The position of cells is marked by the nuclear dye TOPRO. (F) Merged image of panels A-C. (G) Gel mobility shift assays of oligonucleotide probes spanning the tandem binding sites at 271, and the single sites at 570 and 640, with the Sd TEA domain (left panels of each pair). Mutations introduced at these sites (right panels of each pair), reduce Sd binding. F, free probe; 1, probe with one Sd TEA domain bound; 2, probe with two Sd TEA domains bound. (H) A wing disc from one of the transformant lines in which the 271 doublet, and 570, 640 single Sd sites were mutated in the context of fragment EC.

case of *kni*$^{ri[1]}$, to demonstrate whether this point mutation was responsible for the loss of enhancer function. We used the EX *kni*$^{ri[53j]}$ PCR fragment to make a *lacZ* construct, confirmed the sequence of the mutant construct, generated four independent transformants, and tested these flies for *lacZ* expression in third instar wing discs. We found that *EX*(*ri*^{53j})-*lacZ* mutant wing discs fail to express *lacZ* in the L2 primordium (Fig. 2H), strongly suggesting that this single base pair mutation is responsible for the observed L2 vein loss phenotype observed in *kni*$^{ri[53j]}$ flies. One notable difference between the *EX*(*ri*^{53j})-*lacZ* and *EΔri*^{1}-*lacZ* mutant wing discs is that in the point mutant construct (*ri*^{53j}), *lacZ* expression is lost selectively in the L2 primordium and in the weaker posterior stripe (note the normal levels and pattern of expression in extreme anterior and posterior regions of the disc, Fig. 2H), while in the deletion mutant construct (*EΔri*^{1}), reporter gene expression is eliminated throughout the wing disc (Fig. 2G).

As noted above, the *kni*$^{ri[53j]}$ mutant is also associated with a deletion of ≈50 bp in fragment R, which lies approximately 15 kb 5′ to fragment E within predicted intron sequences of a putative transcription unit (see *kni* locus map, Fig. 1E). As the ubiquitous expression of this transcription unit is not affected in *kni*$^{ri[53j]}$ mutants (K. L., unpublished observations) and fusion of fragment R with *lacZ* does not result in gene expression in the L2 primordium, we have no evidence to suggest that this aberration has any relevance to the L2 vein loss phenotype of *kni*$^{ri[53j]}$ mutants.

Analysis of the *kni*$^{ri[M3]}$ allele indicates that this mutation involves an insertion and possibly rearrangements within fragment E. One breakpoint of the *kni*$^{ri[M3]}$ allele falls within the 1.4 kb EX minimal L2 enhancer element (Fig. 1F). Because this is a relatively weak *ri* allele and is associated with a complex lesion, we did not characterize the nature of this rearrangement further.

The wing selector protein Scalloped binds to the L2 enhancer and is required for its activity

The 4.8 kb L2 enhancer fragment E and truncated derivatives (fragments EX and EC) drive reporter gene expression specifically in the wing pouch. Recent work has shown that gene expression in the wing primordium is controlled by Sd, which functions with Vestigial (Vg) to define wing identity (Kim et al., 1996; Kim et al., 1997; Halder et al., 1998; Simmonds et al., 1998; Guss et al., 2001; Halder and Carroll, 2001). We tested whether Sd might similarly be required to activate endogenous *kni* expression by generating clones of cells homozygous for a strong hypomorphic allele of *sd* using FLP-FRT mediated mitotic recombination (Golic, 1991). In *sd* clones, β-galactosidase expression driven by the *E-lacZ* element is reduced (Fig. 3D). In clones falling along the future wing margin, however, reporter gene expression is unaffected (not shown; see Discussion). These results indicate that Sd plays an in vivo role in activating *kni* expression within the wing blade.

Because Sd, a TEA-domain protein, functions as a direct transcriptional regulator of several key developmental genes expressed in the wing disc (Halder et al., 1998; Guss et al., 2001), we tested whether Sd binds to the *kni* L2 enhancer. We searched for specific Sd DNA binding sites in the 0.69 kb EC activation domain using DNAse I footprinting and gel shift analysis (Fig. 3G and data not shown) and identified five sites bound by the Sd TEA domain, which conform well to the known Sd consensus binding site and consist of a tandem doublet of Sd binding sites and four single binding sites (Fig. 3A). The four single Sd sites are removed by the 252 bp deletion in the *kni*$^{ri[1]}$ allele, however, none of them covers the *kni*$^{ri[53j]}$ point mutation. To determine whether Sd plays a direct role in activating the L2 enhancer, we mutated a subset of these Sd binding sites in the context of the *EC-lacZ* construct, transformed these mutated constructs into flies and stained for *lacZ* expression in third instar wing discs. We found that mutation of the doublet at 271 in combination with the two single sites at 570 and 640 resulted in a complete loss of *lacZ* expression in the wing disc (Fig. 3H). These results indicate that Sd is required for activation of *kni* expression in the wing disc.

kni$^{ri[1]}$ mutants can be rescued by *kni*, *knrl* or the downstream target gene *rho*

Having shown that the minimal L2 enhancer is mutated in several independently derived *ri* alleles (*kni*$^{ri[1]}$, *kni*$^{ri[M3]}$, and *kni*$^{ri[53j]}$), we wished to know whether driving expression of either the *kni* or *knrl* genes with this element could rescue the *ri* vein-loss phenotype. We addressed this question using the conditional GAL4/UAS expression system of Brand and Perrimon (Brand and Perrimon, 1993). We created transgenic flies that carry fragment E driving GAL4 expression (L2-

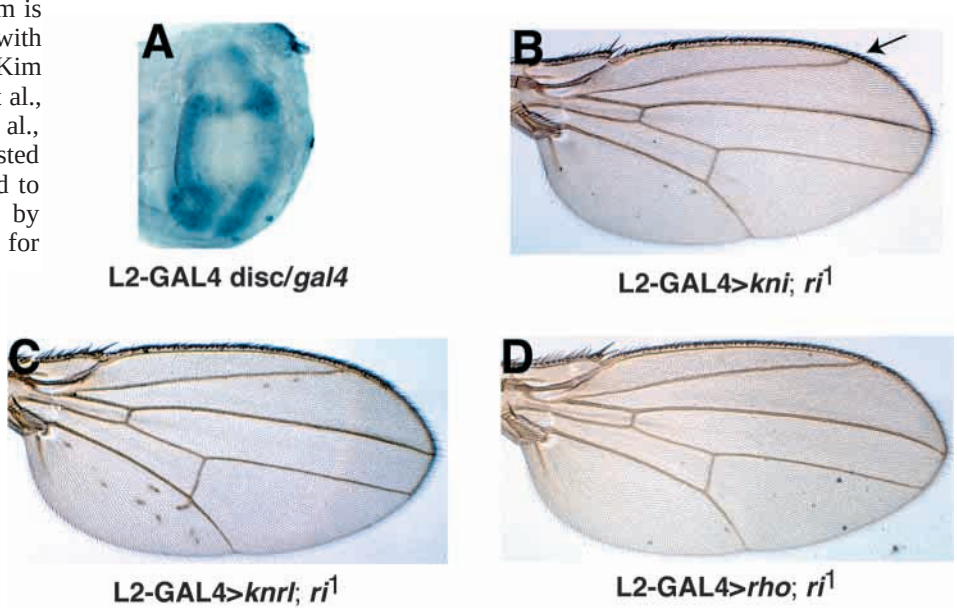

Fig. 4. Rescue of L2 in *kni*$^{ri[1]}$ by targeted gene expression in the L2 primordium. (A) *GAL4* expression in an L2-*GAL4* mid-third instar larval wing disc. (B) Rescue of the L2 vein (arrow) in an L2-*GAL4>UAS-kni*; *kni*$^{ri[1]}$ male adult wing. (C) Rescue of the L2 vein in an L2-*GAL4>UAS-knrl*; *kni*$^{ri[1]}$ male adult wing. (D) Rescue of the L2 vein in an L2-*GAL4>UAS-rho*; *kni*$^{ri[1]}$ male adult wing.

GAL4) and used this driver to activate expression of UAS-*kni* or UAS-*knrl* transgenes in the L2 primordium of *kni*$^{ri[1]}$ mutants. As expected, *GAL4* RNA is expressed in the L2 primordium of L2-GAL4 wing discs (Fig. 4A). We placed this L2-GAL4 driver and the UAS-*kni* and UAS-*knrl* transgenes into the *kni*$^{ri[1]}$ mutant background and generated flies of the genotype L2-GAL4>UAS-*kni*; *kni*$^{ri[1]}$/*kni*$^{ri[1]}$ (Fig. 4B) and L2-GAL4>UAS-*knrl*; *kni*$^{ri[1]}$/*kni*$^{ri[1]}$ (Fig. 4C). These flies have fully restored L2 veins that resemble wild-type L2 veins in that the bulk of the vein is formed on the ventral surface of the wing.

Since *rho* is known to play a critical role in activating Egfr signaling in all vein primordia, we also determined whether expression of this downstream effector of the *kni* locus could bypass the requirement for *kni* in *kni*$^{ri[1]}$ mutants. We found that L2 formation is indeed rescued in L2-GAL4>UAS-*rho*; *kni*$^{ri[1]}$/*kni*$^{ri[1]}$ flies (Fig. 4D). As in the case of rescue by *kni* or *knrl*, the rescued L2 vein forms primarily on the ventral surface of the wing.

Misexpression of genes expressed in veins other than L2 alters L2 development

As mentioned above (see Introduction), several genes that are important for vein development are expressed in veins other than L2. For example, the vein- and sensory organ-promoting gene *ara* is expressed in the odd numbered veins (L1, L3, and L5), the proneural genes *ac* and *sc* are expressed in L1 and L3, and the Notch ligand Delta is expressed in L1 and L3-L5, but not in L2. We were interested to know whether it is necessary to exclude expression of these genes from the L2 primordium for normal L2 development. To address this question, we misexpressed UAS-transgene copies of these genes in the L2 primordium with the L2-GAL4 driver and examined the L2 vein in adult wings. In each case, we observed defects in L2 development. Misexpression of the lateral inhibitory signal Delta in L2-GAL4>UAS-*Dl* flies results in modest truncation of the L2 vein in females (Fig. 5A) and almost entirely eliminates the L2 vein in sibling males (Fig. 5B), which are the consistently more severely affected sex. The near elimination of L2 development in severely affected *Dl*-misexpressing males is accompanied by a loss of *rho* expression in the L2 primordium of third instar larval wing discs (Fig. 5D, compare with wild-type *rho* expression in Fig. 5C). Misexpression of the proneural gene *sc* in the L2 primordium of L2-GAL4>UAS-*sc* flies results in L2 veins covered with ectopic bristles (Fig. 5E). The great majority of these ectopic bristles are strictly confined to the L2 primordium, consistent with the double label experiments (Fig. 2C) indicating that the L2-enhancer element drives gene expression precisely in the L2 primordium. In addition, ectopic bristles form sporadically in a broad posterior domain of the wing, which derives from a region of the wing disc in which the L2 enhancer element is also expressed in a weak diffuse pattern (Fig. 2B,D). Finally, misexpression of the *ara* gene in L2-GAL4>UAS-*ara* flies causes a reproducible thinning of the L2 vein (Fig. 5F). Cumulatively, these results underscore the importance of excluding expression of non-L2 vein genes from the L2 primordium.

The L2-GAL4 driver can be used as a tool to dissect gene function and range of action

As discussed above, the L2-GAL4 driver activates UAS transgene expression precisely in the L2 primordium. The ability to drive highly localized expression of genes in a non-essential tissue with L2-GAL4 suggests that this driver could be used as an effective tool to dissect the function and range of action of various genes. For example, since

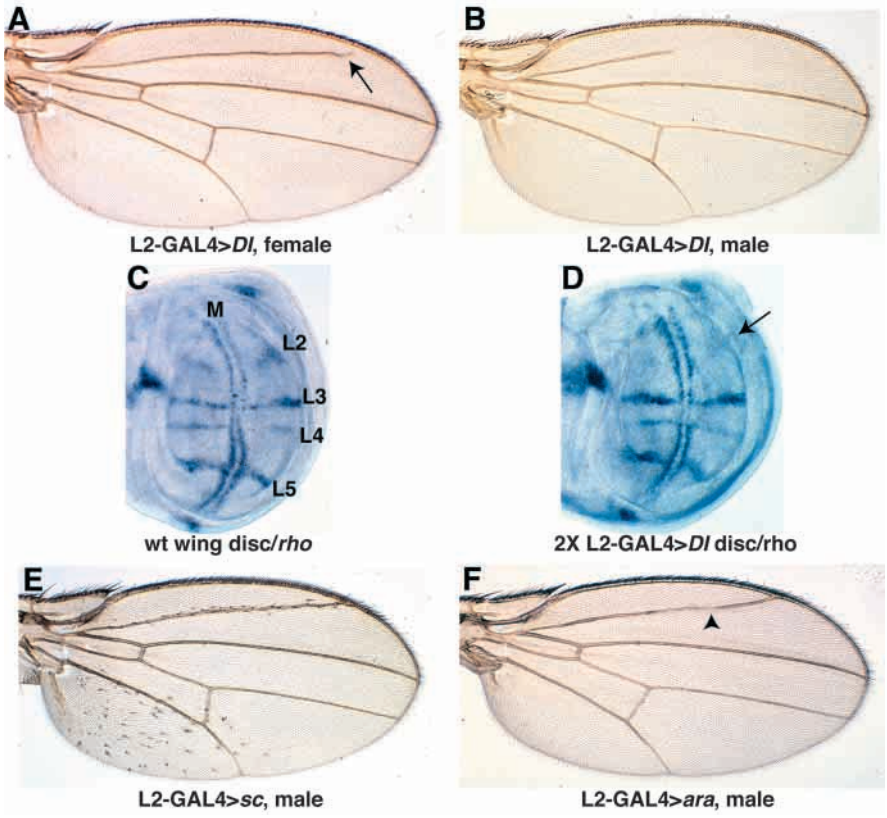

Fig. 5. Misexpression of non-L2 vein genes in the L2 primordium alters L2 development. (A) An *L2-GAL4>UAS-Dl* adult female wing. The distal tip of the L2 vein is typically missing (arrow). (B) An *L2-GAL4>UAS-Dl* adult male wing. The L2 vein is consistently truncated to this severe extent. (C) *rho* expression in a wild type mid third larval instar disc. Vein primordia L2-L5 and the margin are indicated. (D) *rho* expression in a 2X *L2-GAL4>UAS-Dl* mid-third instar larval wing disc is lost selectively in the L2 primordium (arrow). (E) An *L2-GAL4>UAS-sc* adult male wing. Ectopic bristles are largely confined to the L2 vein as well as to a broad posterior domain, which presumably corresponds to cells in *L2-GAL4* wing discs expressing high (L2) or moderate (posterior domain) levels of GAL4 (e.g. see Fig. 4A). (F) An *L2-GAL4>UAS-ara* adult male wing. Note that the width of the L2 vein is reduced in the middle (arrowhead). This thinning of L2 by misexpression of *ara* is a consistent phenotype that we have not observed when we misexpress the related gene *caup* in the same pattern (not shown).

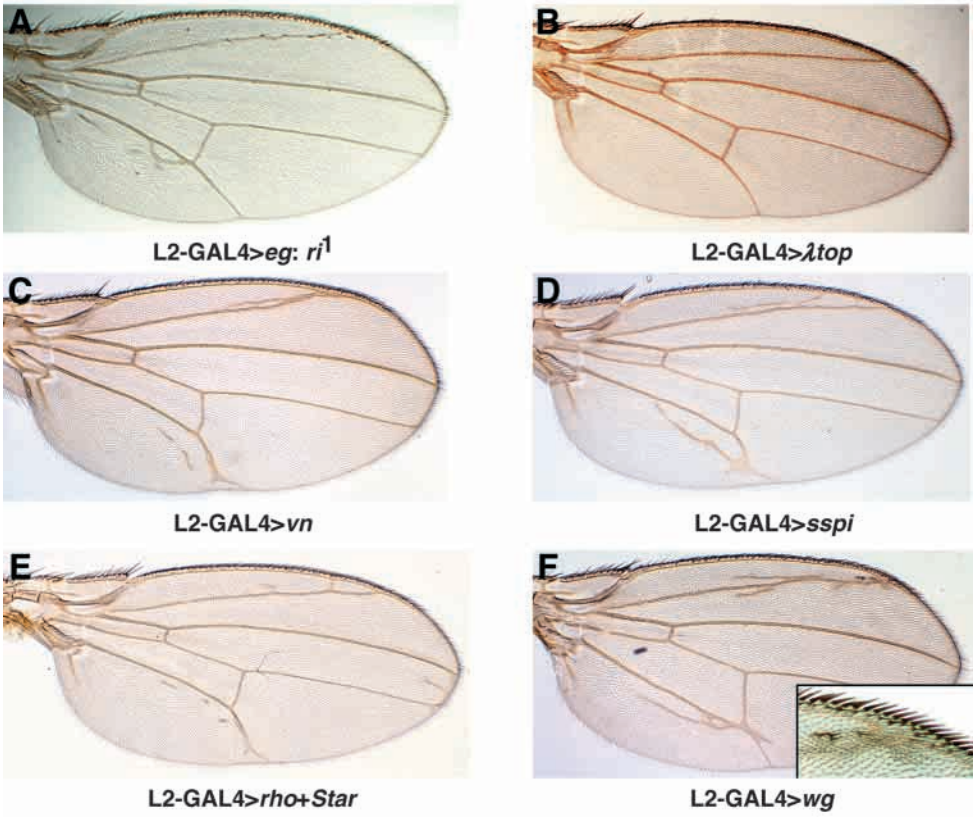

Fig. 6. Use of the L2-GAL4 expression system to assay gene function in the wing. (A) Rescue of the L2 vein in an L2-*GAL4>UAS-eg*; *kni*$^{ri[1]}$/*kni*$^{ri[XT2]}$ male adult wing. Note the ectopic sensory bristles along the reinstated L2 vein. (B) An *L2-GAL4>UAS-λtop* adult male wing. (C) An *L2-GAL4>UAS-vein* adult male wing. Note the minor degree of ectopic venation (e.g. anterior to L2 and at the junction of L2 with the margin). (D) An *L2-GAL4>UAS-secreted-spitz* adult female wing. Note the ectopic vein running parallel and anterior to L2. (E) An *L2-GAL4>UAS-rho+UAS-Star* wing. Note patches of ectopic vein material anterior to L2. (F) An *L2-GAL4>UAS-wingless* adult male wing. Note change in shape of the margin, ectopic vein material both anterior and posterior to L2 and ectopic bristles near the junction of L2 with the margin (magnified in insert).

the *eagle* (*eg*) gene encodes a steroid hormone receptor closely related to Kni and Knrl (Rothe et al., 1989) we wondered whether this gene could substitute for *kni* or *knrl* in rescuing *kni*$^{ri[1]}$ mutants. *eg* is involved in neuroblast specification during embryogenesis (Higashijima et al., 1996; Dittrich et al., 1997; Lundell and Hirsh, 1998), but is not expressed in the wing pouch (data not shown) and is not known to play a role in development of the wing proper. We found that, as in L2-GAL4>UAS-*kni*; *kni*$^{ri[1]}$/*kni*$^{ri[1]}$ individuals, L2 formation is rescued in L2-GAL4>UAS-*eg*; *kni*$^{ri[1]}$/*kni*$^{ri[XT2]}$ flies, however, this restored 'L2' vein is decorated with a line of ectopic sensory bristles (Fig. 6A). As the same ectopic bristles are observed in L2-GAL4>UAS-*eg* individuals (data not shown), this phenotype can be attributed to misexpressing *eg* in the L2 primordium. By employing the L2 expression system it should now be possible to map the domain in Eg that is responsible for inducing ectopic bristles using chimeric Eg/Kni receptors.

The L2 expression system should also be useful in distinguishing cell-autonomous from non cell-autonomous gene activity. As a test of this idea we compared the phenotypes resulting from driving expression of an activated form of the Egfr (λtop) in the L2 primordium versus expression of the secreted Egf-like ligands Vein (Schnepp et al., 1996) and sSpitz (Schweitzer et al., 1995; Schnepp et al., 1998). Consistent with λtop acting in a cell autonomous fashion to promote vein versus intervein development, expression of this gene in the L2 primordium of L2-GAL4>UAS-*λtop* wings (Fig. 6B) had no effect on the development of the L2 vein or neighboring intervein cells. In contrast, when secreted Egf ligands were expressed in a similar fashion in L2-GAL4>UAS-*vn* wings (Fig. 6C) or in L2-GAL4>UAS-*sspi* wings (Fig. 6D), we observed ectopic veins that formed 2-3 cell diameters away from the L2 vein. Similarly, when we co-expressed *rho* and *Star* in the L2 primordium (e.g. in L2-GAL4>UAS-*rho*; UAS-*Star* wings; Fig. 6E), which also generates a potent secreted Egfr promoting activity (Guichard et al., 1999), we observed ectopic veins that were displaced from the endogenous L2 vein by a few cells. The ability of secreted ligands, but not the *λtop* construct, to induce ectopic vein formation at a distance cannot be attributed simply to the latter being a weaker activator of Egfr signaling since UAS-*λtop* is considerably more effective at generating ectopic veins than UAS-*vn* when other wing-GAL4 drivers are used (e.g. MS1096-GAL4 or CY2-GAL4, data not shown).

We also observed a non cell-autonomous activity of the Wingless (Wg) ligand in L2-GAL4>UAS-*wg* wings (Fig. 6F), which had ectopic veins displaced from the endogenous L2 as well as ectopic marginal bristles near the intersection of L2 with the margin. These ectopic marginal bristles always formed on the appropriate surface of the wing (Fig. 6F, insert), suggesting that this phenotype may result from the inappropriate activation of the wing margin genetic program.

DISCUSSION

An important step in understanding the link between patterning and morphogenesis is to identify key *cis*-regulatory elements that lie at the interface of these sequential processes. Formation of the L2 vein provides a potential paradigm for connecting patterning initiated by a morphogen (e.g. a centrally positioned

BMP activity gradient) to the activation of a vein differentiation program in a narrow stripe of cells in the wing imaginal disc (e.g. controlled by *kni*/*knrl*). In this study we have identified a *cis*-acting regulatory element that drives expression of the *kni* locus in the L2 primordium. We found that three distinct mutations causing L2 vein truncations alter this regulatory element and that in at least two of these cases the mutations disrupt the function of the enhancer. Dissection of this enhancer element indicates that a locally provided activating signal acts in conjunction with broadly distributed activating (*sd* dependent) and repressive systems to limit activation of the L2 enhancer to a sharp stripe of cells in the wing imaginal disc. We also found that excluding expression of non L2 vein genes from the L2 primordium is essential for normal L2 development.

ri mutants are L2-enhancer specific regulatory alleles of the *kni* locus

The results described in this study demonstrate definitively that *ri* mutations are regulatory alleles of the *kni* locus disrupting the function of a *cis*-regulatory enhancer element that drives gene expression in the L2 primordium of wing imaginal discs. A crucial line of evidence supporting this conclusion is that mutant versions of the L2 enhancer incorporating either the 252 bp deletion present in *kni*$^{ri[1]}$ or the single base pair substitution present in *kni*$^{ri[53j]}$ eliminate the ability of this element to direct gene expression in the L2 primordium. In addition, it is possible to completely rescue the vein-loss phenotype of *kni*$^{ri[1]}$ by expressing either the UAS-*kni* or UAS-*knrl* transgenes with an L2-GAL4 driver. Consistent with activation of *rho* being one of the key effectors of *kni*/*knrl* function, it is also possible to rescue the L2 vein-loss phenotype of *kni*$^{ri[1]}$ by expressing a UAS-*rho* transgene in L2, although rescue is less complete and penetrant than that observed with UAS-*kni* or UAS-*knrl*.

The isolation of the L2 enhancer also addresses an unresolved question regarding the basis for the failure of *Df(3L)kni*$^{ri[XT2]}$ and *Df(3L)kni*^{FC82} to complement (Lunde et al., 1998). As these two deletions have endpoints that break within the same 1.7 kb *Eco*RI fragment (indicated by the dashed vertical lines in Fig. 1E), one explanation for the failure of complementation could be that this 1.7 kb fragment contains the L2 enhancer. The alternative explanation for the *ri* phenotype of *Df(3L)kni*$^{ri[XT2]}$/*Df(3L)kni*^{FC82} is that transvection (Lewis, 1954; Geyer et al., 1990; Pirrotta, 1999), which normally occurs between regulatory and coding region alleles of the *kni* locus (Lunde et al., 1998), is interrupted by the large divergent deletions that have little if any overlap. Since the 4.8 *Eco*RI fragment containing the L2 enhancer maps nearly 10 kb upstream of the 1.7 kb *Eco*RI fragment, and a *lacZ* fusion construct (abd-*lacZ*, Fig. 1E) containing the 1.7 kb *Eco*RI fragment does not drive any gene expression in the wing disc (Lunde et al., 1998), the latter hypothesis that *Df(3L)kni*$^{ri[XT2]}$ and *Df(3L)kni*^{FC82} are unable to engage in effective transvection is the most likely explanation for the failure of these two mutations to complement.

Global activation, regional repression and localized induction restrict L2-enhancer activity to a narrow stripe of cells

The results of these and previous studies of L2 vein initiation lead to a model in which localized vein inductive signaling acts against a background of regional repression and global wing-specific activation (Fig. 7A). Previous genetic experiments have suggested a model in which *sal*-expressing cells produce a short-range L2 inducing signal (X) to which *sal*-expressing cells themselves cannot respond (Sturtevant et al., 1997; Lunde et al., 1998). According to this 'for export only' signaling model, the signal X diffuses into adjacent anterior cells and activates expression of the *kni*/*knrl* genes in the L2 primordium, in much the same fashion as Hh, expressed from the refractory posterior compartment, activates expression of target genes such as *dpp* or *ptc* in the anterior compartment (reviewed by Bier, 2000). Based on the analysis of the L2 enhancer element described in this study, the 252 bp region deleted in *kni*$^{ri[1]}$ mutants may contain response element(s) to this putative factor X. It is worth noting, however, that *lacZ* expression is lost in all cells of the wing disc (posterior and circumferential cells as well as the L2 primordium) when these sequences are deleted from the 4.8 kb fragment. This observation suggests that sequences mediating more general activation (e.g. Sd binding sites, see below) may also be contained within this 252 bp region. The sequence surrounding the single nucleotide alteration in *kni*$^{ri[53j]}$ mutants (C596A), however, is a particularly intriguing candidate for mediating the L2-specific activation of *kni* since introducing this mutation in the context of the minimal 1.4 kb enhancer leads to selective loss of *lacZ* expression only in the stripe of cells adjacent to the *sal* domain.

The hypothetical transcription factor X′ (Fig. 7B) that binds to the region surrounding the *kni*$^{ri[53j]}$ point mutation and mediates the inductive signal X presumably collaborates with the more generally required wing selector Sd (Halder et al., 1998; Guss et al., 2001), since mutation of four of the Sd binding sites (the doublet and two single sites) in the L2 activation domain completely eliminates enhancer activity in the wing disc. Clonal analysis with a hypomorphic *sd* allele also indicates that *sd* is required for high-level expression of the full 4.8 kb L2 enhancer element in the wing disc. It is notable that the reduction in *lacZ* expression in these clones is not as dramatic as the complete loss of L2 activity observed when Sd binding sites in the activation domain are mutated. There are several possible explanations for this discrepancy. Firstly, the *sd* mutation used in these experiments is a hypomorphic allele and therefore has residual activity. Unfortunately, stronger *sd* alleles produce even smaller viable clones in the wing disc and thus were not used. Secondly, as only small clones can be generated, they must typically have been produced with only two or three intervening cycles of cell division. Consequently, the *sd*$^{-}$ cells may still contain functional levels of wild type Sd (protein perdurance). Another possibility is that other activators can partially substitute for Sd, at least in certain regions of the wing. Based on the absence of L2 activity when Sd binding sites are mutated and the reduction in L2 activity in *sd*$^{-}$ hypomorphic clones, we conclude that Sd plays an important role as an activator of the L2 enhancer. These results support the view that Sd functions as a general transcriptional activator of genes expressed in the wing field.

Repression also plays a key role in restricting L2 enhancer expression to a narrow stripe of wing disc cells. It has been shown previously that *salm* and *salr*, which are expressed

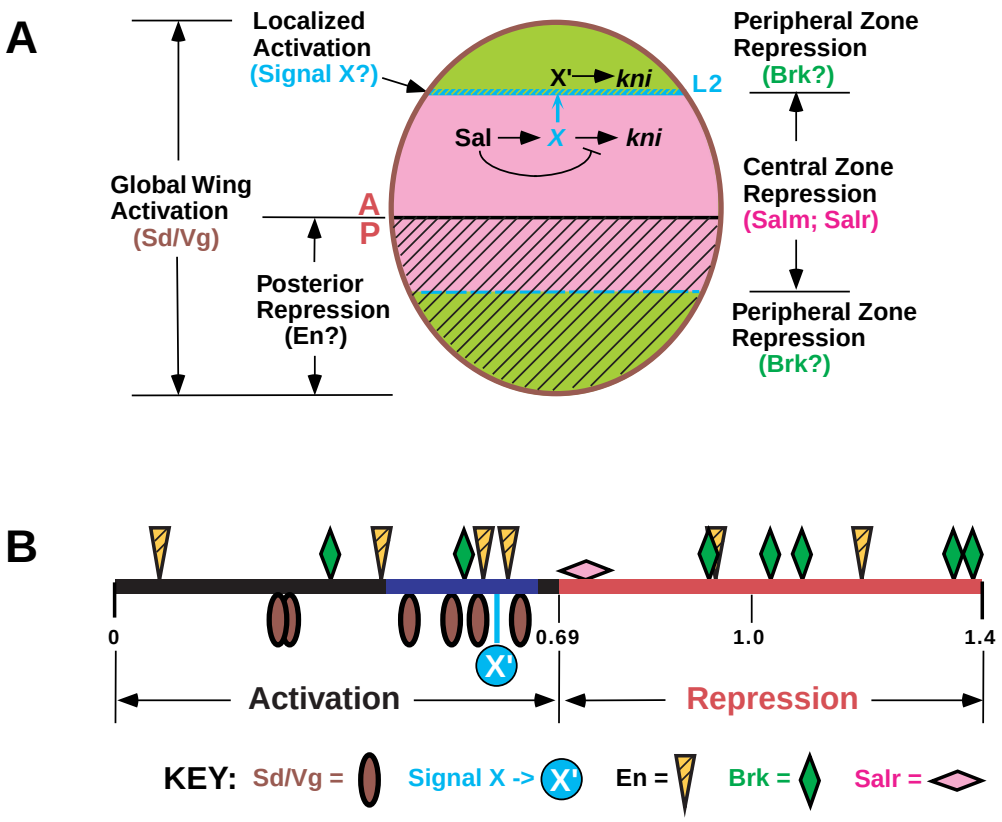

Fig. 7. A model for L2 initiation: global activation, regional repression and local induction. (A) Activation of *kni* expression in the L2 primordium as a consequence of the action of patterning genes initiates morphogenesis of the L2 vein. Analysis of the *kni*/*knrl* locus L2 enhancer element reveals that this element is activated in a narrow stripe by a combination of global wing specific activation, regional repression, and localized signal mediated activation. Global wing-specific activation is provided at least in part by Sd/Vg (this study). Regional repression is mediated either directly or indirectly by Salm/Salr (Sal) in central wing cells (Lunde et al., 1998), and by a repressor(s) acting in peripheral wing cells (green domain) – possibly Brk (this study). In addition, a short range signal X (blue), which is produced by Sal-expressing cells (pink domain) is required to induce *kni/knrl* expression (blue line) in cells just anterior to *sal*-expressing cells. Since *kni*/*knrl* expression is activated in cells just anterior to the *sal* expression domain, but not in posterior cells adjacent to the *sal* domain (dashed blue line), there must be either a repressor (e.g. En?) acting in the posterior compartment (hatched) or an activator acting in the anterior compartment together with X′ which provides this AP specificity in *kni/knrl* activation. The AP compartment border is indicated (solid black line). (B) The *kni*/*knrl* L2 enhancer can be subdivided into separate domains mediating at least certain components of activation (black region) and repression (red region). Global wing-specific activation, which requires Sd/Vg function (this study), is mediated by some combination of the empirically determined Sd binding sites (brown ovals) of which there are four single sites and one double site in the activation region. The action of the hypothetical signal X is presumably mediated by a transcription factor X′, which binds to sequences in the activation domain. The binding site for factor X′ (blue circle) may include sequences containing the single nucleotide mutated in the $kni^{ri[53j]}$ mutant (C596A) since L2 enhancer activity is selectively lost in the L2 primordium of these flies. This site is also eliminated in the 252 bp deletion in the $kni^{ri[1]}$ mutant (dark blue region), as are additional sequences required for activity of the enhancer throughout the wing (e.g. four single Sd sites). The repression domain contains five predicted binding sites for the repressor Brk (green diamonds), two sites for En (hatched yellow triangles), and one site for Salr (pink diamond). Since the reporter gene expression driven by the activation region alone is repressed in central cells, this fragment must also contain some yet unidentified binding sites for Salm, Salr, or some other factor mediating the repressive effect of Salm/Salr.

strongly in the central region of the wing, repress expression of *kni* and *knrl* (Lunde et al., 1998), although low levels of *sal* may also be required to activate *kni* expression (de Celis and Barrio, 2000). In the current study we also find evidence for repression of L2-enhancer activity in peripheral wing disc cells abutting those expressing high levels of *sal*. Truncation of the minimal 1.4 kb (fragment EX) L2 enhancer element results in reporter gene expression expanding to fill the anterior and posterior regions of the wing pouch in a pattern complementary to that of *sal*. The region deleted from the EX fragment contains several consensus binding sites for Brinker (Fig. 3B, Fig. 7B) (Rushlow et al., 2001; Zhang et al., 2001), which may mediate this repression since the pattern of *brinker* (*brk*) expression in the wing pouch (Campbell and Tomlinson, 1999; Jazwinska et al., 1999) is very similar to that of *lacZ* expression driven by the 0.69 kb fragment EC. One way to integrate the action of the localized inductive signal X→X′ pathway with that of abutting central and peripheral repressive factors is to propose that the signal-dependent activator X′ can overcome the repressive action of the peripheral inhibitor but not repression by Salm/Salr (see legend to Fig. 7). One feature common to several prominent signaling pathways is that activation of the pathway converts a resting repressor into a transcriptional activator (Barolo and Posakony, 2002). In the *kni* L2 enhancer, activation of the hypothetical signal X pathway may relieve repression by a heterologous repressor since the putative activator (i.e. X′) and repressor (e.g. Brk?) sequences in the L2 enhancer are separable. One example of a signaling pathway that functions by relieving inhibition by a heterologous repressor is the recent report of Egfr signaling inactivating repression by Suppressor of Hairless (Su(H)) (Tsuda et al., 2002**)**. In addition to central and peripheral repression in the wing disc, there may also be a repressor in posterior compartment cells (e.g. En, Fig. 7A,B) to prevent activation of the L2 enhancer in cells posterior to the *sal* expression domain. Perhaps the 4.8 kb L2 enhancer element lacks some sites for this putative repressor since *E-lacZ* drives significantly higher levels of gene expression in the posterior

stripe than that observed for endogenous *kni* or *knrl* expression.

Expression of non-L2 vein genes must be excluded from the L2 primordium

All longitudinal veins share several morphological characteristics such as being composed of densely packed cells on both the dorsal and ventral surfaces of the wing that secrete a thickened cuticle. The primordia of all longitudinal veins also express the *rhomboid* gene, which is required for activating the Egfr pathway in vein but not in intervein cells. In addition to these shared properties, each vein can be distinguished by expression of other vein genes (Biehs et al., 1998). For example, vein L2-specific characteristics include: expression of *kni* and *knrl*, lack of Delta expression, lack of *caup*/*ara* expression, and lack of *ac*/*sc* expression. Given that all veins are ultimately quite similar morphologically, it is relevant to ask whether the differences in gene expression patterns observed in different veins are important.

In this study, we examined the necessity of excluding expression of non-L2 vein genes in the L2 primordium by forcing expression of genes such as *Dl*, *ara*, *ac* and *sc* in L2 and asking whether this manipulation had any impact on L2 development. These experiments strongly suggest that exclusion of non-L2 vein genes from the L2 primordium is indeed important since misexpression of each of these genes resulted in abnormal L2 development. The phenotypes resulting from misexpressing non-L2 vein genes in the L2 primordium can largely be reconciled with the normal functions of these genes. For example, forced expression of *Dl* leads to loss of L2, consistent with suppression of vein formation by Notch signaling. The ectopic bristles that form strictly along the L2 vein in wings misexpressing *ac* or *sc* are also consistent with the neural promoting function of proneural genes. It is less clear why misexpression of the *ara* gene causes thinning of the L2 primordium, since this gene normally activates expression of the vein-promoting gene *rho* in odd numbered veins and *ac* and *sc* in the L3 primordium. Perhaps expression of a gene normally involved in development of the odd numbered dorsal veins is somehow incompatible with development of the ventral L2 vein.

The L2 expression system provides a localized assay for gene function in the wing

The sharp stripe of gene expression driven by L2-GAL4 can also be used as a rapid assay to test the function or range of action of various genes in the wing. For example, in the case of the Egfr pathway, it is possible to distinguish components exerting cell-autonomous versus non cell-autonomous functions. In line with the fact that activation of the Egfr pathway in the wing leads to the formation of veins, L2-GAL4-driven expression of the secreted ligand sSpi generates ectopic veins in the neighborhood of L2 while expression of the equally potent constitutively active λtop form of the EGF receptor does not result in a non-autonomous phenotype. The L2 expression system may also be used to compare the functions of related genes such as the nuclear receptors Kni, Knrl, and Eg. Although these three transcription factors share nearly identical DNA binding domains and can all rescue the vein-loss phenotype of *ri* mutants, they differ in that misexpression of *eg* in the L2 primordium induces the formation of ectopic bristles along L2. This distinct activity of *eg* may relate to its normal embryonic function in directing cell fate choices in the CNS. Using the L2 expression system as an assay, it should be straightforward to map the domain in *eg* that is responsible for its neural inducing capacity by constructing chimeric molecules composed of different domains of Eg and Kni. Finally, the observation that L2-driven expression of *Dl* leads to a highly penetrant loss of L2 in males and a consistently weaker phenotype in females provides the basis for a modifier screen to identify mutations that either suppress the phenotype in males or enhance the phenotype in females. Some of the modifier loci identified in such a screen, which are not specifically involved in Notch signaling, may encode components of the hypothesized factor X→X′ pathway.

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