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Notch signalling mediates segmentation of the *Drosophila* leg

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SUMMARY

The legs of *Drosophila* are divided into segments along the proximodistal axis by flexible structures called joints. The separation between segments is already visible in the imaginal disc as folds of the epithelium, and cells at segment boundaries have different morphology during pupal development. We find that Notch is locally activated in distal cells of each segment, as demonstrated by the restricted expression of the *Enhancer of split mβ* gene, and is required for the formation of normal joints. The genes *fringe*, *Delta*, *Serrate* and *Suppressor of Hairless*, also participate in Notch function during leg development, and their expression is localised within the leg segments with respect to segment boundaries. The failure to form joints when Notch signalling is compromised leads to shortened legs, suggesting that the correct specification of segment boundaries is critical for normal leg growth. The requirement for Notch during leg development resembles that seen during somite formation in vertebrates and at the dorsal ventral boundary of the wing, suggesting that the creation of boundaries of gene expression through Notch activation plays a conserved role in co-ordinating growth and patterning.

Key words: Leg development, Notch signalling, *Drosophila melanogaster*, Imaginal disc

INTRODUCTION

The Notch signalling pathway participates in a vast array of cell fate decisions during the development of multicellular organisms. Its roles can broadly be divided into two categories. The first is exemplified by the selection of neural precursors, both in *Drosophila* and other organisms, where Notch prevents competent cells from following a neural fate (Artavanis-Tsakonas and Simpson, 1991; Lewis, 1996). Conversely, the second category of Notch signalling results in cells acquiring specialised characteristics, and hence has been referred to as inductive Notch signalling (Artavanis-Tsakonas et al., 1995). It has been best characterised in the *Drosophila* wing where the activation of Notch at the confrontation between dorsal and ventral wing cells is required for the expression of several genes with critical roles in the growth of the disc (Irvine and Vogt, 1997). Vertebrate limb development also requires Notch and, as in the *Drosophila* wing, this leads to the establishment and maintenance of a domain of gene expression that separates flanking cell populations and co-ordinates their growth (Irvine and Vogt, 1997). Similarly Notch activity regulates the formation and patterning of the somites during mouse development in a manner that suggests it is needed to generate boundaries between the emerging somites (Conlon et al., 1995; Hrabe de Angelis et al., 1997; Khang and Gridley, 1998; Evrard et al., 1998). These examples suggest a common theme, in which localised activation of Notch is responsible for establishing boundaries, that is likely to be of widespread significance during morphogenesis. We are therefore exploring the extent to which morphogenetic defects caused by *Notch* mutations in *Drosophila* are brought about by failure to form stable boundaries of gene expression. One process where the formation of boundaries between homologous structures appears to be important for morphogenesis is in segmentation of the *Drosophila* leg.

Drosophila legs are cylindrical and segmented appendages, with each leg having 9 segments separated by flexible joints (Fristrom and Fristrom, 1993). The joints form at precise positions along the proximodistal axis of the leg, and both the expression pattern of several genes in the leg and the results of regeneration experiments suggest that different positions along the proximodistal axis have different identities (Cohen, 1993). Two signalling molecules, wingless (wg) and decapentaplegic (dpp) play a central role in patterning the leg discs. Their genes are activated in complementary anterior dorsal (*dpp*) and anterior ventral (*wg*) sectors in response to the secreted protein Hedgehog, which is only expressed in posterior cells (Basler and Struhl, 1994). The asymmetry of *dpp* and *wg* expression is maintained by mutual repression (Jiang and Struhl, 1996; Brook and Cohen, 1996; Penton and Hoffmann, 1996) and they act antagonistically to regulate several genes involved in generating differences along the dorsoventral axis (Lecuit and Cohen, 1997). In addition *wg* and *dpp* in combination activate another set of genes involved in proximodistal patterning which are expressed in ring-shape territories in different domains along the proximodistal axis of the leg (Lecuit and Cohen, 1997). It is likely therefore that the proximodistal patterning system initiated by *wg* and *dpp* determines the

localisation of presumptive joints in developing leg discs, but the identity of the gene products mediating this process is unknown.

Although the mechanism underlying joint formation is not understood, the fusion of segments caused by some *Notch* alleles indicates a requirement for Notch signalling (Shellenbarger and Mohler, 1978). Amongst the other components of the Notch pathway there is evidence to suggest that the two transmembrane ligands Delta (Dl) and Serrate (Ser) are required in joint formation, as mutations cause leg-segment fusions similar to Notch (Parody and Muskavitch, 1993; Speicher et al., 1994), but as yet the roles of the other genes have not been examined. Activation of Notch involves proteolytic processing steps that liberate the intracellular domain of Notch which then collaborates with the transcription factor Suppressor of Hairless (Su(H)) to regulate expression of target genes (Weinmaster, 1998). The best characterised targets include a group of related genes forming the *Enhancer of split* gene complex (*E(spl)*) which encode basic helix-loop-helix proteins that act as transcriptional repressors (Jennings et al., 1994; Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Expression of these genes therefore gives an indication of the cells where Notch is active. At the dorsal-ventral boundary of the wing Notch activity is detected and required in the cells that abut the boundary, and the expression of the ligands is maximal in this region with Serrate in the dorsal cells and Delta predominantly in ventral cells (Irvine and Vogt, 1997). Similar high levels of Notch ligands are detected at the developing somite boundaries in the paraxial mesoderm (Hrabe de Angelis et al., 1997). Both these processes also require Fringe, which helps to restrict the interaction between Notch and its ligands (Panin et al., 1997; Fleming et al., 1997; Khang and Gridley, 1998; Evrard et al., 1998).

To investigate the role of Notch signalling in the segmentation of the leg we have used a combination of clonal analysis, mis-expression experiments and molecular markers. We find that Notch is locally activated in rings of cells at the distal end of each leg segment during imaginal development. Furthermore, *Notch* activation at segment boundaries is not only critical for the formation of joints but also affects the growth of each leg segment. Thus as in the wing, the activity of Notch is required in the leg to establish boundaries which act as organising centres for the subsequent growth within leg segments.

MATERIAL AND METHODS

Genetic strains

We used the loss-of-function alleles $l(1)N^{\beta}$ $l(1)N^{3}$, Dl^{M1} (de Celis et al., 1996), the *E(spl)* deficiency $Df(3R)E(spl)^{grob32.2}$ (a deficiency that deletes all seven *E(spl)* bHLH genes; Schrons et al., 1992) and the lethal alleles $Su(H)^{AR9}$ (Schweisguth and Posakony, 1994) and fng^{13} (Irvine and Wieschaus, 1994). Cell markers used for clonal analyses were *forked* (f^{36a}) and *mwh* (Lindsley and Zimm, 1992) and transgenes carrying the *f* wild-type allele inserted in 37A, 77D and 87F (designated P[f$^+$]37A, P[f$^+$]77B and P[f$^+$]87F respectively). The *Minute* alleles to generate M^+ clones were *M(1)15D, M(2)24F, M(3)65D* and *M(3)95A* (Lindsley and Zimm, 1992). We used *lacZ* enhancer-trap lines in *fng* ($fng^{lacZ35UZ-1}$; Irvine and Wieschaus, 1994), *Notch* (N^{lacZ1}; de Celis et al., 1997) *Delta* ($Dl^{lacZP1651}$) and *bigbrain* ($bib^{lacZ6E1}$, J. de C., Tom Weaver and S. J. B., unpublished), which reproduce most aspects of the normal expression of the corresponding genes in the leg. For mis-expression studies we used the UAS lines UAS-Dl (Doherty et al., 1996), UAS-Ser (Speicher et al., 1994), two different insertions of UAS-Nintra (de Celis and Bray, 1997), UAS-Necd (Klein et al., 1997) and UAS-fng (Kim et al., 1995).

Generation of the reporter E(spl)mβ-CD2

A 1.5 kb Psp1406I fragment containing the *E(spl)mβ* promoter and the start-site of transcription was combined with the rat CD2 coding sequence (from CD2/PMTL22; gift from Nick Brown) to generate a fusion in the 5′ untranslated region of the *E(spl)mβ* mRNA. This E(spl)mβ-CD2 fusion construct was excised as a *Kpn*I-*Not*I fragment and ligated into the P-element transformation vector pWhiteRabbit (gift from Nick Brown). Multiple independent *white+* transformant lines were generated and all gave similar patterns of expression which are indistinguishable from the endogenous expression of the *E(spl)mβ* gene.

Generation of mitotic recombination clones

Mitotic recombination was induced by X-rays (dose 1000 R; 300 R/minute, 100 kV, 15 mA, 2 mm aluminium filter). Clones were induced at the interval 48-72 hours after egg laying. This results in a range of clone sizes that varies between clones restricted to only one segment to clones that form the entire anterior or posterior compartments. In all cases leg phenotypes were only detected when mutant clones spanned more than one segment. Mutant clones in the X chromosome were generated in flies of genotype N^{*} $f^{36a}/M(1)15D$, where N^{*} represent $l(1)N^{3}$ and $l(1)N^{ß}$ *Notch* alleles. Mutant clones in the 3R chromosomic arm were generated in males of genotypes f^{36a}*;mwh M(3)95A P[f$^+$]87F Mutant* (M^+ clones), where *Mutant* represent Dl^{M1} or $Df(3R)E(spl)^{grob32.2}$ alleles. $Su(H)^{AR9}$ clones were induced in flies of genotype f^{36a}*; M(2)24F P[f$^+$]37A/*$Su(H)^{AR9}$ and *fng* clones were induced in flies of genotype f^{36a}*;M(3)65D P[f$^+$]77/mwh* fng^{13}. In all cases mitotic recombination proximal to the f^+ insertion produces homozygous mutant clones labelled with the cell marker *f*.

Quantification of phenotypes

Wild-type tarsi (20) or tarsi carrying large $l(1)N^{3}$ (11) or $E(spl)^{grob32.2}$ (18) clones were drawn using a graphic tablet, and their area and length calculated using the program NIH Image 1.61.

Immunocytochemistry and in situ hybridisation

We used rabbit anti-β-galactosidase (Cappel), rabbit anti-Ser (Thomas et al., 1991), guinea-pig anti-Coracle (Fehon et al., 1994), and mouse monoclonals anti-Notch (Fehon et al., 1991), anti-Dl (Kooh et al., 1993), anti-CD2 (Serotech) and anti-β-galactosidase (Promega) antibodies. Secondary antibodies were from Jackson Immunological Laboratories (used at 1/250). Whole-mount in situ hybridisation with digoxigenin-labelled DNA probes was performed as described previously (de Celis et al., 1996). For *fng* we used as probe a 1.9 kb *Eco*RI *fng* cDNA fragment (Irvine and Wieschaus, 1994).

RESULTS

Phenotype of loss-of-function mutations in several elements of the Notch pathway

Drosophila legs consist of a series of segments separated by flexible joints (Fig. 1D). Each segment has a characteristic size, shape and pattern of sensory organs (Held, 1995; von Kalm et al., 1995; Fig. 1A,B). In the leg imaginal disc most segments form concentric rings, with the most distal in the centre of the disc (Fristrom and Fristrom, 1993). The exceptions are the

distal femur and proximal tibia, which are indistinguishable in the larval imaginal disc and only separate during pupariation. This separation occurs through the formation of lateral invaginations that fuse creating two epithelial tubes constricted at the femur/tibia joint (Fristrom and Fristrom, 1993).

When Notch activity is compromised in N^{ts1} larvae during early and late third instar stage, the legs that develop are misshapen with some fusion between femur/tibia (early) and tarsal (late) segments (Shellenbarger and Mohler, 1978). To determine more precisely where Notch activity is required during leg development we analysed the phenotypes produced by clones of cells which have greatly reduced *Notch* function. Two hypomorphic *Notch* alleles, $l(1)N^3$ and $l(1)N^\beta$ were used, because unlike *Notch* null alleles they do not autonomously prevent cell proliferation (de Celis et al., 1996). In all clones of these *Notch* alleles that span a joint, no joint tissue is formed by mutant cells (Fig. 1C-F). However, wild-type cells that are in contact with *Notch* mutant cells are able to form joint structures, so that incomplete joints form at normal locations in mosaic joints (Fig. 1F). With the limitations of the cell markers we used, the effects of these two *Notch* mutations on joint differentiation appears to be cell autonomous. In addition, all clones in the anterior-ventral compartment of the femur and tibia interfere with the separation between the distal femur and the proximal tibia (Fig. 1E). Legs carrying large anterior or posterior *Notch* clones are always shorter that their normal counterparts, and mosaic tarsal segments have a 25% reduction in area and a 30% reduction in length compared with wild-type controls (see Materials and Methods). Finally, clones restricted to only one leg segment do not affect the size of this segment or the overall morphology and size of the leg. Therefore it is only when clones span the joint that both defects in joint formation and global effects on the growth of the leg are seen. To distinguish which elements of the Notch pathway are required during leg development we generated clones of homozygous mutant cells, using lethal alleles in *fng*, *Dl* and *Su(H)* as well as a deficiency of the *E(spl)* complex. Lethal *Ser* alleles can survive into adults and they have a low frequency of joint fusions (Speicher et al., 1994).

The phenotype of *Dl* and *Su(H)* mosaics are similar to each other and, like *Notch*, result in a failure to make joints when mutant cells are in the position where a joint should have formed (Fig. 2A,B,G). Again, the wild-type cells near the clones can still form joints, but the length of the leg is reduced when the mutant clones are large and span more than one segment. In contrast, mutant cells homozygous for a deficiency that removes the *E(spl)bHLH* genes form normal joints even when they span more than one segment and are characterised by the differentiation of a vast array of ectopic sensory organs (Fig. 2C-D). These develop without intervening epidermal cells, indicating that *E(spl)* is required for the lateral inhibition mechanism that allows the spacing between sensory organs. The larger clones cause a slight reduction in the overall size of the leg (12% in area and 8% in length), but it is likely that these effects are due to the differentiation of ectopic sensory organs rather than direct effects on growth.

Cells mutant for *fng* also result in fusions between segments. However, these effects are position dependent. Thus, with clones spanning the boundary between the femur and tibia the phenotypes are indistinguishable from those of *Notch* and *Su(H)*, resulting in a fusion of these two segments and shortening of the leg (Fig. 2E), whereas in more distal segments we can only detect defects in the joint between the proximal two tarsal segments (Fig. 2F). The fact that *fng* is important in leg segmentation suggests that boundaries similar to the wing dorsal-ventral boundary are being created in at least some of the presumptive joints.

The expression of several components of the Notch pathway is restricted to segment boundaries during leg development

In the developing wing the localised activation of Notch can be detected by the activation of certain target genes such as

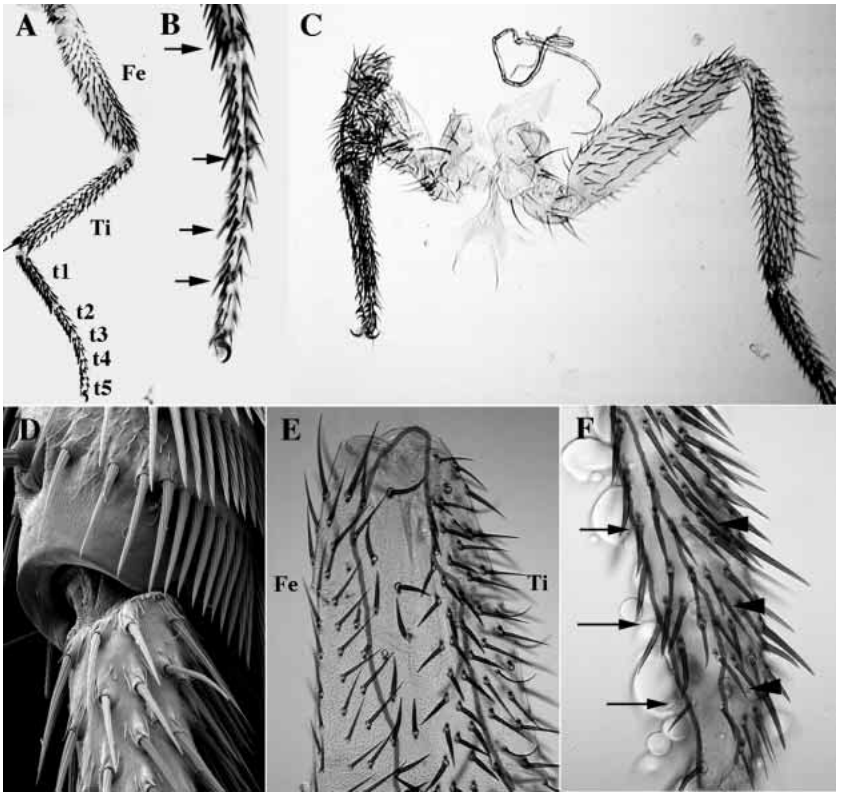

Fig. 1. Requirements for *Notch* during leg segmentation. (A) Wild-type mesothoracic leg showing the seven distal segments: femur (Fe), tibia (Ti) and five tarsal segments (t1-t5). (B) Higher magnification of t2-t5 with arrows indicating the joints. (C) Metathoracic legs with a large $l(1)N^3\ f^{36a}$ posterior clone in the left leg that causes foreshortened femur and tibia, and partial fusion of tarsal segments. (D) Scanning electron micrograph of the joint between the tibia and the first tarsal segment, showing the specialised cuticular differentiation at the joint. (E) Anteroventral $l(1)N^3\ f^{36a}$ clone causing the fusion between the distal femur (Fe) and proximal tibia (Ti). (F) $l(1)N^3\ f^{36a}$ clone in the tarsi causing autonomous disappearance of the joints between t4/t5, t3/t4 and t2/t3 (arrows). Note the presence of incomplete joints (arrowheads) in the wild-type compartment.

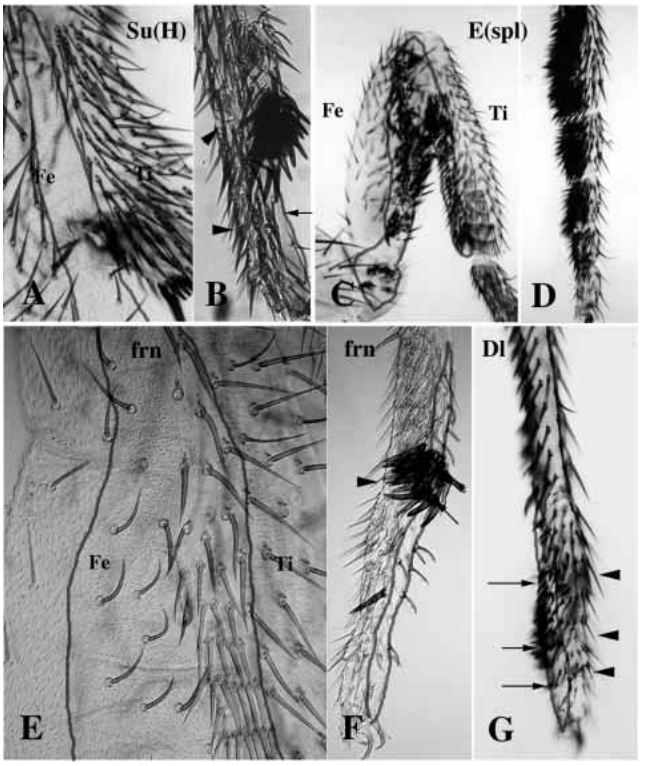

Fig. 2. Leg phenotypes caused by mutations in *Su(H), E(spl), fng* and *Dl*. (A-B) *Su(H)*^{AR9} clone in the anterior ventral compartment causes the fusion between distal femur (Fe) and proximal tibia (Ti) (A), and the failure to form the joint by mutant cells (B, arrows). *Su(H)* clones (thin line) do not differentiate the external component of sensory organs, and are identified by the presence of naked cuticle. Normal joint tissue is marked by arrowheads. (C-D) *E(spl)*$^{grob32.2}$ clones differentiate ectopic sensory organs, but the separation between femur and tibia (C) and the formation of joints between tarsal segments (D) is not affected. (E) *fng*^{13} anterior-ventral clone, labelled with *mwh* and *f*, causes fusion between distal femur (Fe) and proximal tibia (Ti). (F) Incomplete fusion between tarsal segments caused by anterior *fng*^{13} clone. The joint between tarsal segments 1 and 2 is indicated by an arrowhead. (G) *f*^{36a} *Dl*^{M1} clone in the tarsi results in the fusion between tarsal segments (arrows). The remnants of some joints are still recognisable (arrowheads).

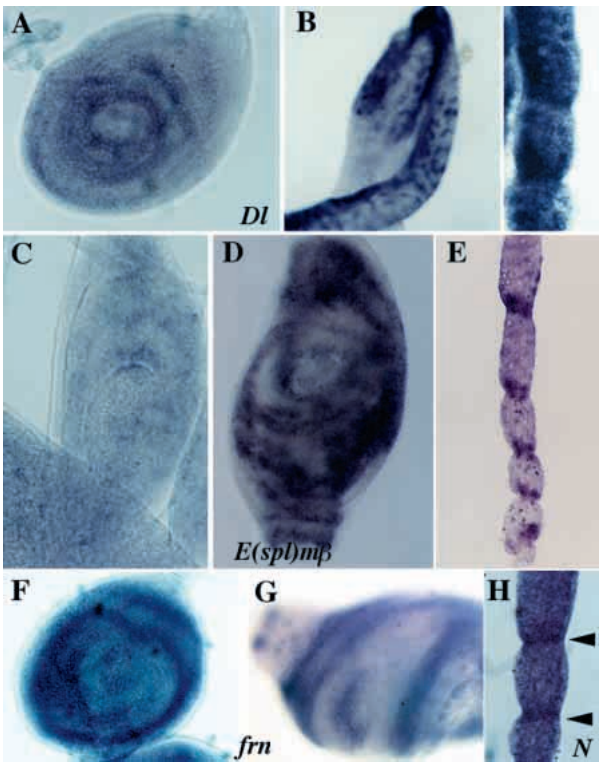

Fig. 3. Expression of *Notch*, *Dl*, *E(spl)mβ* and *fng* during imaginal and pupal leg development. (A-B) Expression of *Dl* RNA in a leg imaginal disc from third larval instar (A) and in everting legs 24 hours after puparium formation (APF) (B). In the disc maximal levels of *Dl* are detected in a series of concentric rings. In the pupal leg *Dl* expression is maximal in the junction between the femur and tibia, in proneural clusters, and at the distal end of each tarsal segment (B, right). (C-E) Expression of *E(spl)mβ* mRNA in early third instar leg disc (C), in evaginating pupal leg disc (D) and in pupal legs 24 hours APF (E). *E(spl)mβ* mRNA is expressed in rings of cells localised at segment boundaries throughout third instar and pupal stages of leg development. (F-G) Expression of *fng* RNA in leg discs in the third larval instar (F) and 2 hours APF (G) is maximal in broad ring-shaped domains that correspond to the tibia and first tarsal segment in the distal appendage. (H) Expression of *Notch* RNA in legs at 24 hours APF is widespread, but higher levels are detected in cells at the positions where joints are forming (arrowheads).

E(spl) (de Celis et al., 1996) and *vestigial* (Kim et al., 1996). Furthermore, the domains of expression of Dl and Ser are important in creating this localised activation of Notch (Irvine and Vogt, 1997). We therefore examined the expression of *Ser*, *Dl*, *fng*, *Notch* and *E(spl)mβ* during leg development. Heterogeneities in the expression of all these genes were detected in the third instar imaginal disc, where *Dl* and *E(spl)mβ* RNA are expressed in narrow concentric rings (Fig. 3A,C). In evaginating leg discs (0-4 hours APF) and in pupal legs, when the separation between leg segments becomes more evident, *E(spl)mβ* expression is localised to a ring of distal cells in each leg segment (Fig. 3D,E), suggesting that its larval expression also defines the distal end of each segment. The expression of *fng* is also restricted, and is only detected in several broad rings localised to the presumptive tibia and first tarsal segment, and in two groups of distal cells in the fifth tarsal segment that could correspond to the presumptive claws (Fig. 3F-G). At this stage, we can not detect any heterogeneity in the expression of *Notch* RNA (not shown), but by 24 hours after puparium formation the levels are higher in the places where the joints are being formed (Fig. 3H) which appear to be the same cells where *E(spl)mβ* is expressed (Fig. 3E). At these later stages, *Dl* also accumulates in rings of cells located at the distal end of each segment and at the separation between the femur and tibia, as well as in many clusters of cells that correspond to developing sensory organs (Fig. 3B).

Expression of *E(spl)* genes is dependent on Notch activity and hence the localisation of *E(spl)mβ* mRNA to rings of cells in the imaginal and pupal leg disc indicates that there are high levels of Notch activation in the distal-most set of cells in each segment. To determine more precisely the relationship between the *E(spl)mβ*-expressing cells and the expression of other components of the Notch pathway, we generated a reporter gene in which 1.5 kb of genomic DNA upstream of *E(spl)mβ*

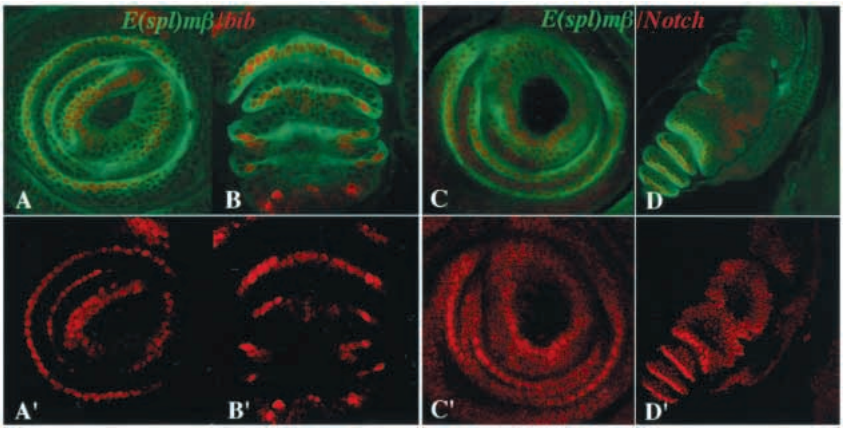

Fig. 4. Localisation of Notch activity at leg segment boundaries. (A-B) Co-expression of $bib^{lacZ6E1}$ (red) and E(spl)mβ-CD2 (green) in third instar (A) and 6 hours APF (B) leg discs. A′ and B′ show the corresponding red channels. In $bib^{lacZ6E1}$ β-galactosidase is expressed in a ring of cells localised at the distal end of each leg segment which coincide with the cells where E(spl)mβ-CD2 is detected. (C-D) Co-expression of E(spl)mβ-CD2 (green) and N^{lacZ1} (red) in third instar (C) and 6 hours APF (D) discs. C′ and D′ show the corresponding red channels, β-galactosidase expression in the N^{lacZ1} line is maximal at segment boundaries.

was used to drive expression of a rat cell surface protein, CD2. As a landmark for the segment boundaries we used an enhancer trap in the *bib* gene, bib^{lacZ}, which is expressed at higher levels in single-cell wide rings at the distal end of each leg segment during both larval and pupal development (Fig. 4).

The expression of *E(spl)mβ-CD2* is localised to a narrow ring, 1-2 cells wide, which coincides with the cells expressing bib^{lacZ} (Fig. 4A-B) and with cells that have higher levels of *lacZ* expression in the N^{lacZ1} *enhancer* trap line (Fig. 4C-D). The expression of N^{lacZ1} at the dorsoventral boundary and at vein-intervein boundaries is dependent on Notch activity itself (de Celis et al., 1997). Thus the coincidence of *Notch*, *E(spl)mβ* and *bib* expression indicates that high levels of Notch activation during imaginal leg development are restricted to the most distal cells of each segment. The accumulation of Notch ligands is also localised within the developing leg segments with the highest levels of Dl and Ser detected in a narrow stripe of cells localised proximally to those expressing bib^{lacZ} both in the larval imaginal disc and at pupal stages (Figs 5A-B, 6A). In agreement with the phenotypes we obtained, expression of

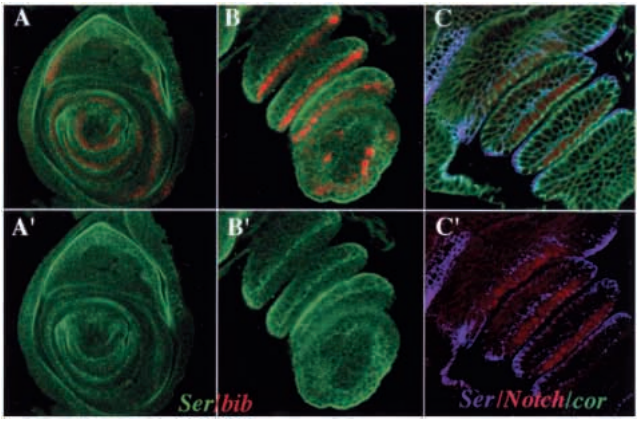

Fig. 5. Relationship between Ser expression and the segment boundaries. (A,B) Ser (green) is maximal in a ring of cells localised proximal to cells expressing $bib^{lacZ6E1}$ (red) in leg discs from third instar larvae (A) and 6 hours APF (B). A′ and B′ are single channel images showing the accumulation of Ser. (C) Expression of N^{lacZ1} (red), Ser (blue) and Coracle (green). Anti-Coracle allows visualisation of the cell membranes. The stripe of Ser-expressing cells is immediately proximal to the maximal expression of N^{lacZ1} at the segment boundary.

fng, detected using a *fng* enhancer trap line, is maximal in the tibia and first tarsal segment. Within these segments it appears to be highest in the regions where bib^{lacZ} and *E(spl)mβ-CD2* are not expressed (Fig. 6C-D). Later, in evaginating discs, low levels of *fng-lacZ* expression are also observed in distal tarsal segments (Fig. 6D). During the formation of the wing dorsoventral boundary *fng* is co-expressed with Ser (Kim et al., 1995), and appears to prevent Notch activation by Ser in dorsal cells (Panin et al., 1997; Fleming et al., 1997). It is likely that *fng* localisation in the legs also contributes to the restriction of Notch activity, and may be important in ensuring that activation of Notch only occurs in the distal cells in the segment.

Temporal evolution of *E(spl)mβ* and *bib* expression in developing leg discs

The appearance of *E(spl)mβ* and *bib* expression in rings of cells should be an early indication of the subdivision of the leg disc into separate segments. Their expression develops progressively during the third larval instar, but because few molecular markers of individual segments have been described we can only establish a limited correspondence between individual rings and leg segments in early stages of imaginal development. E(spl)mβ-CD2 is first detected in second instar leg discs, before any indication of segmentation, in the most proximal cells of the leg epithelium (Fig. 7A-C). Later, in early third instar discs, a novel ring of E(spl)mβ-CD2 develops in the centre of the disc, in the domain where the transcription factor Apterous is expressed (Fig. 7D). The expression of *apterous* is restricted to the cells of the fourth tarsal segment in late third instar and pupal discs (Cohen, 1993; Fig. 7L). Therefore, it appears that the first segment boundary to be formed separates the presumptive tarsal segments 4 and 5. Subsequently, novel rings of both *E(spl)mβ* and *bib* expression develop close to this first central ring (Fig. 7E,J). Most of these are included within the domain of Distal-less expression (Fig. 7I-J), suggesting that they correspond to the developing boundaries between the tibia and t1 and between the tarsal segments t1 to t4. At later stages at least four tarsal segments can be identified by rings of *E(spl)mβ* and *bib* expression, and in addition novel domains of expression develop in the proximal region of the disc (Fig. 7F,K). These observations suggest that the boundaries between presumptive leg segments develop progressively, the first boundaries form in early-mid third instar larvae and correspond to the most distal segments. This temporal evolution is compatible with the observation that

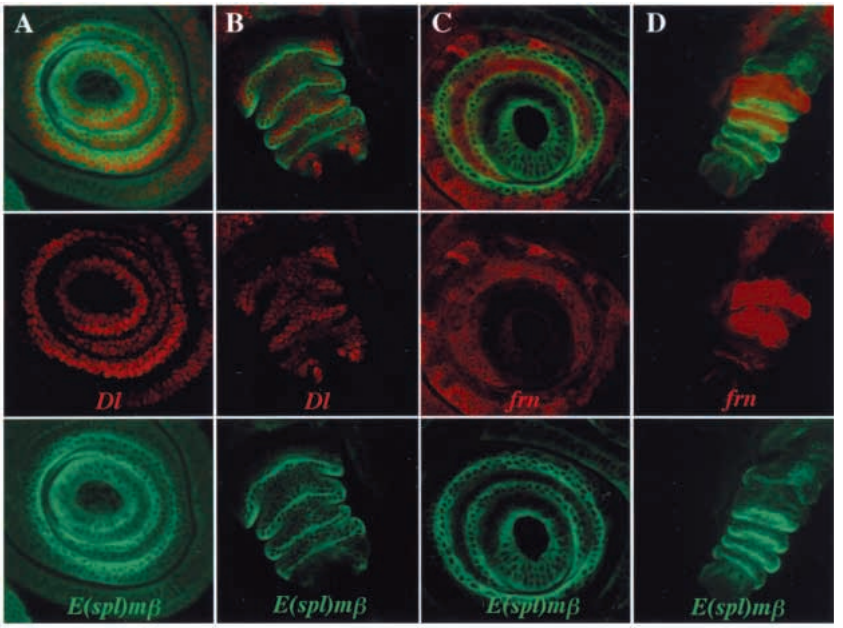

Fig. 6. Relationship between segment boundaries and expression of *Dl* and *fng*. (A-B) Accumulation of E(spl)mβ-CD2 (green) occurs in cells adjacent to those with highest levels of $Dl^{lacZP1651}$ expression (red). (C-D) Complementary expressions of E(spl)mβ-CD2 (green) and fng^{lacZ} (red) are detected in some leg segments. Maximal expression of *fng* is observed in the tibia and first tarsal segment, but lower levels of expression are also apparent in other tarsal segments. Leg discs are from third instar larvae (A,C) or 6 hours APF (B,D).

early leg discs forced to differentiate prematurely exclusively form structures that correspond to distal segments (Schubiger, 1974).

Consequences of mis-expression of elements on the Notch pathway in the leg

To further characterise the relevance of restricted Notch activation during leg morphogenesis, we manipulated the expression of several components of the Notch pathway using the GAL4 system (Brand and Perrimon, 1993). In most of these experiments we used the driver line dpp-GAL4 (Morimura et al., 1996) which is expressed in an anterodorsal sector of leg discs with some weaker anteroventral expression also detectable (see Fig. 9A). Expression of Necd, a dominant negative form of the Notch protein, using this driver line disrupts the joints between tarsal segments, leading to shortened legs (Fig. 8C,G,J). In contrast, expression of the intracellular domain of Notch (Ni), which has ligand-independent Notch activity, causes the formation of ectopic joint structures. The strength of these phenotypes depends on the UAS-Nintra line used. Thus, ectopic expression using dpp-GAL4 and a weak UAS-Nintra insertion (Nintra12.1) results in normal-size legs, which only develop partial joint-like structures, particularly in the tibia and first tarsal segment (Fig. 8D,H). The ectopic joints are incomplete, being restricted to the dorsal side of the leg where expression of GAL4 is highest. When we used a stronger UAS-Nintra line (UAS-Nintra79.2) in combination with the same driver, the legs were extremely abnormal in morphology and both the tibia and femur were bifurcated by an abnormal proximal-distal fold (Fig. 8K,L). In addition the connections between tarsal segments were also affected, with many joint structures appearing in abnormal positions (data not shown). These results demonstrate that Notch activity is both necessary and sufficient to trigger joint formation in leg cells.

Overall the effects produced by ectopic Dl and Ser are similar, the altered morphology of the resulting legs includes both fusion of segments and ectopic joints. However there are positional differences in the way the ligands exert their effects. Thus, the strongest effects of mis-expressing Dl are observed in the tarsal segments, where joint formation is perturbed

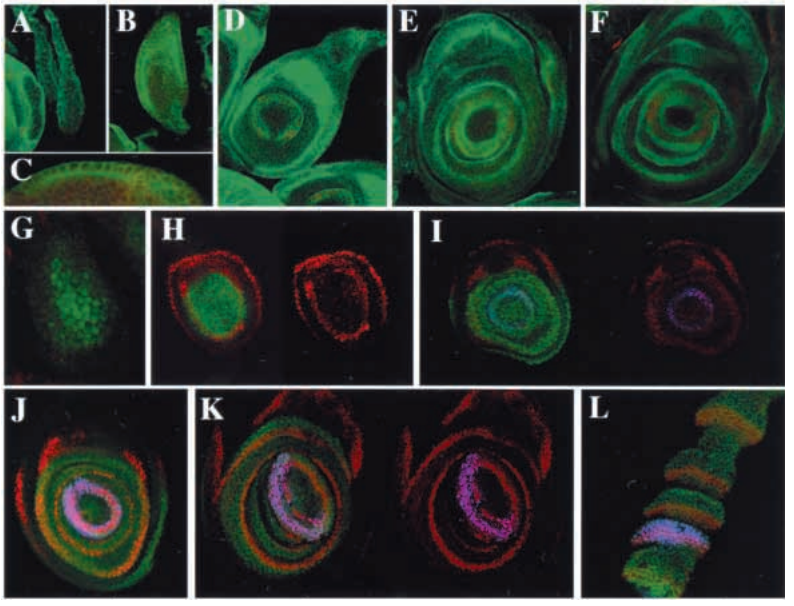

Fig. 7. Temporal evolution of *E(spl)mβ* and *bib* expression in leg discs. (A,B) Expression of E(spl)mβ-cd2 (green) in early (A) and late (B) second instar leg discs. (C) Higher magnification of the proximal cells expressing E(spl)mβ-cd2 in second instar discs. (D-F) Expression of E(spl)mβ-cd2 (green) and *apterous* (*ap-lacZ*, red) in leg discs in early (D), middle (E) and late (F) third instar larvae. (G-L) Expression of *bib-lacZ* (red), Distal-less (green) and Apterous (blue) in leg discs in second (G) early third (H, I), late (J,K) discs and in pupal legs 10 hours after puparium formation.

resulting in foreshortened fused tarsi (Fig. 8A,I). This resembles *Notch* loss-of-function phenotypes suggesting that the levels or position of Dl expression are interfering with normal Notch activity. In addition, an abnormal structure forms at the junction between the first and second tarsal segments, which seems to consist of a partial perpendicular joint (Fig. 8I). The strongest effects of Ser mis-expression are suggestive of dominant negative effects, as the tibia is foreshortened and forms abnormal joints with the femur and tarsi (Fig. 8B,F). In addition incomplete ectopic joints can be observed at low frequency in distal tarsal segments (not shown). Thus, the phenotypes indicate that both activation and repression of Notch occurs when high levels of Notch ligands are expressed. It is likely that the differential effects of misexpression of Dl and Ser are related to the distribution of *fng*, because the strongest dominant negative effects of Ser occur in the tibia, where *fng* expression is maximal, and those of Dl occur in distal tarsal segments, where *fng* is absent or expressed at low levels. Similar effects occur when the ligands are expressed in the wing using the GAL4 system, where the outcome is in part determined by interactions between Notch and Fng (Jonsson and Knust, 1996; de Celis and Bray, 1997; Klein et al., 1997; Panin et al., 1997).

There is a good correlation between the adult phenotypes observed after mis-expression of *Notch*, *Dl* and *Ser* and the expression of bib^{lacZ}, both in larval and pupal leg discs. Thus, ectopic expression of *Necd* always eliminates the dorsal (and occasionally the ventral) side of each ring of bib^{lacZ} expression in all tarsal segments (Fig. 9D-E) and *Ni* has the opposite effect causing an extra dorsal stripe of bib^{lacZ} expressing cells (Fig. 9B-C). In addition, ectopic expression of Ser leads to both activation and repression of bib^{lacZ}. For example novel, proximal-distal stripes of bib^{lacZ} expression are detected in the distal tarsal segments (Fig. 9F). The effects of Necd, Ni and Ser on bib^{lacZ} expression are observed from the stage when bib^{lacZ} expression is first detected, suggesting that Notch activity is required at the time when joint development between leg segments is initiated.

DISCUSSION

In developmental processes involving cell fate decisions between competent cells, such as neurogenesis, Notch activation appears to prevent cells from responding to cell-fate promoting signals (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). In other developmental processes, such as wing, vertebrate limb and somite development, Notch activity is important for establishing/maintaining boundaries that separate developmental territories (Irvine and Vogt, 1997). Here we demonstrate that it is in this latter role that Notch is required during *Drosophila* leg development, where Notch is activated in the distal-most cells in each leg segment. In the absence of Notch the segments are fused and the legs foreshortened, suggesting that segment boundaries influence the growth of the leg segments, analogous to the dorsal-ventral boundary in the wing. Further similarities arise from the involvement of both Ser and Dl in the establishment of the joints, and from the fact that the E(spl)bHLH proteins, which play a central role during neurogenesis but not in the dorsal-ventral boundary (de Celis et al., 1996), do not seem to be essential during leg segmentation.

Role of Notch in joint formation

The effects on joint development of reducing *Notch* or *Su(H)* function appear cell autonomous; only mutant cells fail to form joint structures. The adjacent wild-type cells develop normally so that partial joints are formed. In addition Notch activity is

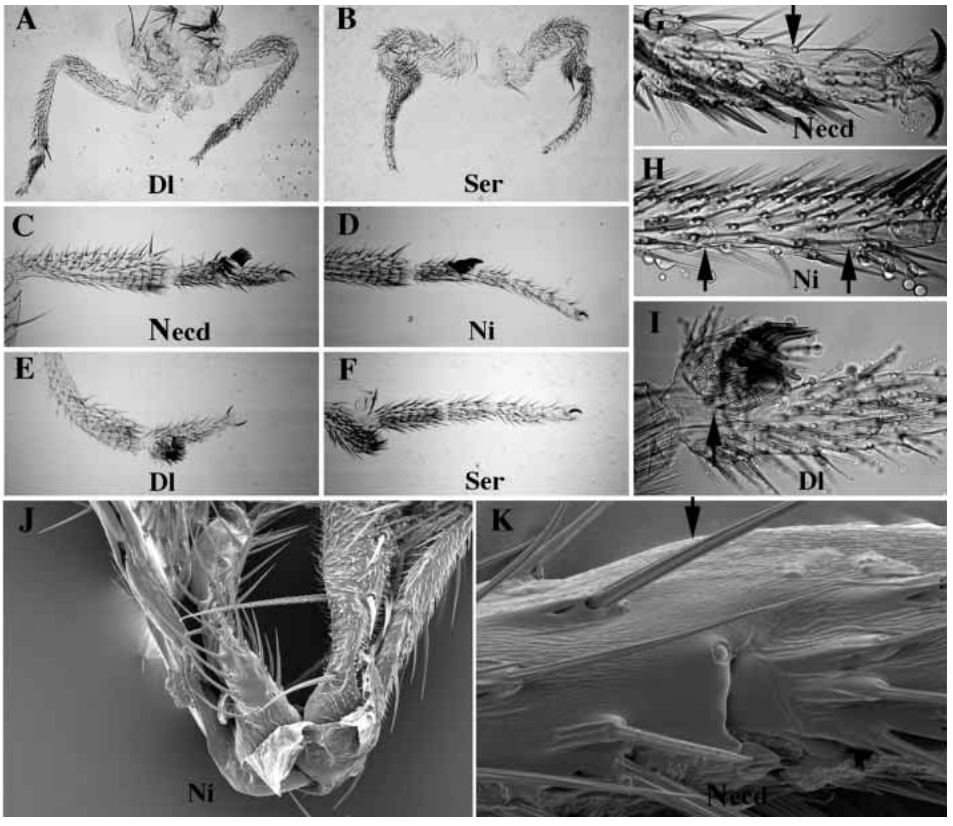

Fig. 8. Effects of ectopic expression of Notch, Dl and Ser on leg development. (A-F) Leg phenotypes resulting from ectopic expression in the *dpp* domain (dpp-Gal4) of Dl (Dl; A,E), Ser (Ser; B,F), dominant negative Notch (Necd; C) and weak activated Notch (UAS-$Ni^{12.1}$; D). Ectopic expression of Dl and Ser affect mainly the tarsal segments and tibia respectively. (G) Higher magnification of tarsi from dpp-Gal4/UAS-Necd flies showing the partial disappearance of joints (arrow). (H) Ectopic joint formation in dpp-Gal4/UAS-$Ni^{12.1}$ (arrows). (I) High magnification of the tarsi of dpp-Gal4/UAS-Dl flies showing the abnormal joint between t1 and t2, that runs along the proximal-distal axis (arrow). (J,K) Scanning electron micrograps of legs resulting from ectopic expression in the dpp domain of UAS-$Ni^{79.2}$ (J) and UAS-Necd (K).

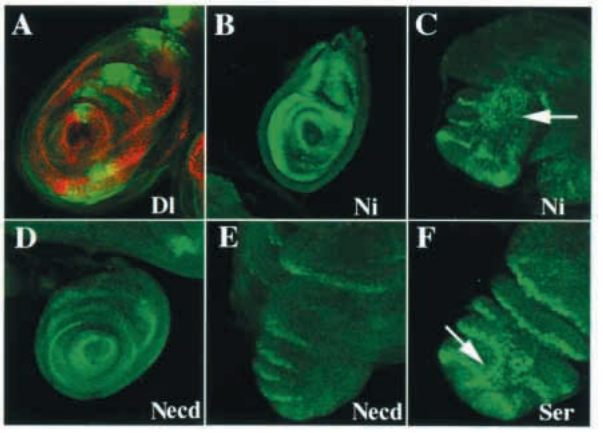

Fig. 9. Mis-expression of Notch, Ser and fng causes modifications in bib expression indicative of disrupted segmentation. (A) Expression of Dl driven by dpp-Gal4 (green) is at higher levels in a dorsal sector, but is also present in the opposite ventral sector. (B-C) Expression of activated Notch (dpp-Gal4/UAS-Ni) causes ectopic expression of $bib^{lacZ6E1}$ in the dorsal side of larval (Ni, B) and pupal (Ni, C) leg discs. (D-E) Expression of $bib^{lacZ6E1}$ is disrupted in dpp-Gal4/UAS-Necd leg discs from larvae (Necd, E) and in 6 hours APF pupal discs (Necd, E) There is a reduction/disappearance in $bib^{lacZ6E1}$ expression in the dorsal side of the developing disc. (F) In dpp-Gal4/UAS-Ser pupal leg discs 6 hours APF there is both loss and ectopic (arrow) expression of $bib^{lacZ6E1}$.

sufficient to promote joint formation since ectopic expression of an activated Notch derivative causes the formation of ectopic, albeit incomplete, joints. Although we cannot demonstrate precisely from our mosaic analysis which cells require Notch, the restricted expression of *E(spl)mβ* to distal cells in each developing segment indicates that Notch is activated in the cells at the boundary. This is further supported by the expression of N^{lacZ} in the same cells, since its transcription is positively regulated by Notch activity in other processes (de Celis et al., 1997). Both ligands, Ser and Dl, are expressed maximally in the cells immediately proximal to the E(spl)mβ domain. The combination of high levels of Ser and Dl can act in a dominant negative way to block the ability of cells to receive the Notch signal, so the distribution of the ligands – highest in the adjacent cells and lower at the boundary – could ensure that Notch is only activated in the cells at the boundary. Thus instead of establishing a symmetrical boundary through the deployment of ligands on both sides, the boundary in the leg segment appears to be asymmetrical. In this respect it may be more similar to the somite boundary where both Dl and Ser homologues appear to be expressed in the same population of cells in the posterior of the somite (Hrabe de Angelis et al., 1997).

At both wing and the somite boundaries the deployment of Fringe appears to be critical and it has been proposed to regulate the interactions between Notch and its ligands (Irvine and Vogt, 1997; Evrard et al., 1998; Khang and Gridley, 1998). Although *fng* expression is present in the developing leg it is predominantly detected in two segments, the tibia and first tarsal segment. This correlates with the phenotype of *fng* mutants, which have defects in the femur/tibia and t1/t2 joints. It also correlates with differential effects of mis-expressing *Dl* and *Ser*. At high levels the ligands have dominant negative effects on joint formation, with Ser these occur principally in the tibia where *fng* is expressed whereas with Dl they occur in the tarsal segments which lack *fng*. This is consistent with the proposed effects of *fng* in the developing wing, where it blocks the ability of Ser, but not Dl, to activate Notch (Panin et al., 1997; Fleming et al., 1997; Klein and Martinez-Arias, 1998).

Segment boundaries are required for normal growth of the leg

In contrast to the autonomous effects on joint development, the effects of Notch on growth have a strong component of non-autonomy, because (1) only large clones spanning several leg segments have noticeable effects on leg size and (2) the reduction in leg size observed in these clones involves both the mutant and wild-type regions of the segment. Similar effects have been observed in the wing, where ectopic activation of Notch is able to promote proliferation of surrounding cells producing outgrowths (Diaz-Benjumea and Cohen, 1995; de Celis and Bray, 1997). For this reason it has been proposed that the dorsal-ventral boundary functions as an organising center, since it appears to co-ordinate the development and growth of all the cells in the wing-field. This organising capacity appears to depend on the activation of at least two genes, *vestigial* and *wingless* that co-operate to promote proliferation and patterning of the wing (Kim et al., 1995; Klein et al., 1997). The non-autonomous effects of the segment boundaries of the leg are suggestive of them exerting similar organising activity on adjacent segments, although in the leg there are no clonal restrictions between segments (Steiner, 1976). Furthermore, neither *vestigial* nor *wingless* are expressed at these boundaries so there are as yet no candidates to mediate this process.

Several mutants affect simultaneously the length of leg segments and the formation of intervening joins, indicating that the two processes are inter-related. For example mutations in *four-jointed* disrupt the joint between the second and third tarsal segments and result in a shortened, fused t2/t3 and truncations in the tibia and femur (Villano and Katz, 1995). However in other mutants, such as *dachsous* and *prickle-spiny legs* (*pk-sple*), joint formation and growth appear to be uncoupled. Thus in *dachsous* mutants, the length of the leg is severely reduced but most joints form correctly (Waddington, 1943). In contrast *pk-sple* mutants have additional joints in the middle of tarsal segments t2 to t5 with no change in the growth of the leg (Gubb and Garcia-Bellido, 1982). These phenotypes suggest that the effects of *Notch* on growth and on joint formation ultimately require different factors. In *pk-sple* mutants it would appear that the segment boundaries are initially established correctly allowing normal growth, and that the ectopic joints form later after proliferation is finished. The joints might therefore be considered analogous to the wing margin whose structures begin to be specified late in larval development and can be uncoupled from wing growth in some circumstances (de Celis and Bray, 1997; Klein et al., 1998). Thus, we suggest that segment boundaries play a dual role during leg morphogenesis. First they act as references for growth during imaginal development and second, they organise the formation of the joint by cells at both sides of the segment boundary.

Since the formation of boundaries between developing leg

segments is critical for their normal growth, the mechanisms which govern the sites where segment boundaries will form are of major significance in leg morphogenesis. Ultimately this is likely to be coordinated by the positional information system that establish differences along the proximal-distal axis of the developing leg disc. This system derives from the cooperative effect of *wg* and *dpp* to direct the expression of several transcription factors such as *distal-less* in proximodistal sectors (Lecuit and Cohen, 1997). Some of these transcription factors could subsequently act in combination to bring about the localised positions of Notch activation. Consistent with this hypothesis, the first ring of *E(spl)mβ* and *bib* expression in early third instar discs corresponds to the outer limit of Distal-less expression. The regulation of Notch by spatially restricted transcription factors could occur via effects on ligand expression and would be one mechanism through which patterned expression of transcription factors is translated into effects on growth and morphogenesis. The identification of the genes which directly mediate the effects on growth will be important to understand the relationship between the formation of organising boundaries and growth in both invertebrate and vertebrate development.

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