Development 126, 2993-3003 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV1389

Composite signalling from *Serrate* and *Delta* establishes leg segments in *Drosophila* through *Notch*

S. A. Bishop[1], T. Klein[2], A. Martinez Arias[2] and J. P. Couso[1,*]

[1]School of Biological Sciences, Royal Holloway College, University of London, Egham, Surrey TW20 0EX, UK
[2]Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, UK
*Author for correspondence (e-mail: j.couso@rhbnc.ac.uk)

Accepted 26 April; published on WWW 7 June 1999

SUMMARY

The receptor protein NOTCH and its ligands SERRATE and DELTA are involved in many developmental processes in invertebrates and vertebrates alike. Here we show that the expression of the *Serrate* and *Delta* genes patterns the segments of the leg in *Drosophila* by a combination of their signalling activities. Coincident stripes of *Serrate* and *Delta* expressing cells activate *Enhancer of split* expression in adjacent cells through *Notch* signalling. These cells form a patterning boundary from which a putative secondary signal leads to the development of leg joints. Elsewhere in the tarsal segments, signalling by DELTA and NOTCH is necessary for the development of non-joint parts of the leg. We propose that these two effects result from different thresholds of NOTCH activation, which are translated into different downstream gene expression effects. We propose a general mechanism for creation of boundaries by *Notch* signalling.

Key words: *Drosophila melanogaster*, *Serrate*, *Delta*, *Notch*, Leg development, Signalling

INTRODUCTION

Animal development involves many types of cell communication processes and the molecular bases of some of these processes have been unravelled in recent years. An unexpected finding has been that the same molecular pathways are used to convey messages between cells in vertebrate and invertebrate animals. One of the most pervasive of these signalling pathways is that of *Notch*. The *Notch* (*N*) gene was identified in *Drosophila melanogaster* on the basis of its requirements in multiple developmental processes. *N* encodes the transmembrane protein NOTCH (N), which has been shown to act as a receptor during cell communication (reviewed in Fleming et al., 1997b). In its best characterised role in the development of the nervous system, *N* has been shown to mediate a process of mutual inhibition whereby an excess of cells with a neural potency compete for a smaller number of neural fates. The N ligand or signalling molecule in this process is the product of the *Delta* (*Dl*) gene (reviewed in Muskavitch, 1994).

In other developmental processes, N receives signals from several ligands (Fleming et al., 1997b). In the induction of a patterning centre at the dorso-ventral (DV) boundary of the presumptive fly wing, the product of the *Serrate* (*Ser*) gene acts as a signal to N. In this particular developmental process, the DELTA protein (DL) is involved and also another transmembrane molecule, FRINGE (FNG), which has been proposed to bind N and modulate its signalling (Panin et al., 1997). This multiplicity of ligands stands in contrast with the proposed simpler one-ligand situation during the singling-out of neural precursors (Heitzler and Simpson, 1991; Muskavitch, 1994), but it has been suggested that *Ser* and the *wingless (wg)* signalling pathways also have a role in several aspects of neural development (Axelrod et al., 1996; Couso and Martinez Arias, 1994; Zeng et al., 1998). Studies in vertebrates also show a multiplicity of N ligands, with multiple homologous proteins to N and its ligands DL and the SERRATE protein (SER) being involved in a variety of developmental processes. In these cases, amongst them somite development and sensory organ development, a variety of DL-like and SER-like ligands are at work (reviewed in Robey, 1997). Thus, it would seem that in N-mediated processes, a situation where combinations of ligands are present might be the norm and a DL-N lateral inhibition process the exception. An understanding of the molecular basis and the cellular logic behind the activation of N by several ligands would be of wide relevance, but so far N signalling seems to show variety rather than a constant mechanism.

The molecular basis of the transmission of the DL-N signal is beginning to be understood, and in short involves the activation and cleavage of the N protein and the transport to the nucleus of the *Supressor of Hairless* protein to act as a transcription factor on target genes like the *Enhancer of split (E(spl)* complex (Fleming et al., 1997b; Weinmaster, 1998). However, it is not yet understood how signals from different N ligands are processed by this pathway. In this work we describe the role of N and its ligands SER and DL in the development of the legs of *Drosophila melanogaster*, and propose a mechanism that might be common to other developmental processes in *Drosophila* and vertebrates.

During fly leg development the anlage is divided in concentric segments along the proximal-distal axis (Couso and Bishop, 1998). At the boundaries between these segments, a multicellular pattern feature is defined, the articulated joints (Fristrom and Fristrom, 1993). These joints provide functionality to the legs and are one of the distinct and fundamental features of the Arthropod phylum. Here we show that *N* signalling allocates the presumptive joint areas between segments. Co-expression of *Ser* and *Dl* in a stripe of cells proximal to the future position of the joints signals the adjacent row of cells to express members of the *E(spl)* complex and to become presumptive joint areas. Autonomous self-signalling by *Ser* and *Dl* expressing cells is reduced to low levels by the presence of the putative repressor FNG and by possible dominant negative effects of SER and DL. We postulate that, as in the development of the fly wing margin, *N* signalling in the presumptive leg joints activates the expression of a secondary signalling process in the target cells. This secondary signalling process then drives the subsequent development of the anlage around it to give rise to the articulated joint.

MATERIALS AND METHODS

Flies

Flies were raised on a standard cornmeal medium at 25°C except in temperature-shift experiments, as indicated. For each genotype, a minimum of 20 adult flies (with a total of 120 legs) were analysed under the dissecting microscope, and a minimum of 60 legs were dissected and analysed under the compound microscope.

Alleles and temperature-sensitive combinations

Oregon-R was used as the wild-type stock. *Ser* mutant animals were identified as Tb^+ progeny from a cross Ser^{RX106} *st e* / TM6b × *Df(3R)D605* / TM6b (a deficiency for the *Ser* locus), raised at 18°C (Couso et al., 1995). *Dl* mutants were identified as Tb^+ progeny from a cross of $Dl^{6B37}e$ / TM6b (temperature-sensitive allele) × Dl^{RF} / TM6b raised at 18°C (Parody and Muskavitch, 1993). *Dl* mutant pupal discs for staining with anti-SER were generated by shifting Dl^{6B37} *e* / Dl^{RF} larvae from 18°C to 25°C. *disco* expression in *Ser* and *Dl* mutants was detected in Tb^+ progeny of the following crosses: *disco lacZ* / *disco lacZ; e* Dl^{6B37} / TM6b × Dl^{RF} / TM6b at 18°C, and *disco lacZ* / *disco lacZ*; *e* Ser^{RX106} / TM6b × *Df(3R)D605* / TM6b at 18°C. The *N* temperature-sensitive allele N^{ts1} (Schellenbarger and Mohler, 1975) was crossed to w^a N^{55e11} / FM7a and shifted from 18°C to 25°C (restrictive temperature) to produce adults with a near total loss of *N* function (Couso and Martinez Arias, 1994).

dsh function was studied using dsh^1 mutants. The dsh^1 mutation disrupts the *dsh* protein domain required for cell polarity (Axelrod et al., 1998). dsh^1 mutants were selected as w^+ y^+ individuals from the stock *v* dsh^1 / FM7a.

LacZ reporter lines

Expression of *disco* was detected by monitoring β-gal expression in flies carrying a reporter *lacZ* construct inserted in the *disco* gene (Heilig et al., 1991). *Dl* expression was monitored using a *lacZ* reporter construct in the stock *Dl lacZ* / TM6b (Klein and Martinez Arias, 1998). *E(spl)* expression was monitored using contructs containing 5′ regulatory regions driving the expression of *lacZ* (Kramatschek and Campos-Ortega, 1994). fng^{2B2} *lacZ* is an enhancer trap line inserted at the *fng* locus (J. P. C. and M. I. Galindo, unpublished), and which faithfully reproduces *fng* expression in imaginal discs.

Gal4 and UAS lines

The Gal4/UAS system of Brand and Perrimon (1993) was used to express genes ectopically. *klu*-Gal4 (Klein and Campos-Ortega, 1997) was used to drive ectopic expression of UAS-*Ser* (Speicher et al., 1994) and UAS-Dl^{30B} (Doherty et al., 1996) using homozygous stocks, UAS-*Ser* (on 3rd chromosome) and UAS-Dl^{30B} (on 2nd chromosome). In the case of UAS-*Ser*, pharates and escapers appear at 25°C, whilst with UAS-Dl^{30B} pharates only appear at 18°C.

The expression of *disco* was assayed in the UAS-*Ser* and UAS-Dl^{30B} escapers by X-gal staining in the Tb^+ progeny of the following crosses: *disco lacZ* / *disco lacZ; klu*-Gal4 / TM6b × UAS-Dl^{30B} and *disco lacZ* /+; *klu*-Gal4 UAS-Green Fluorescent Protein (GFP) / TM6b × UAS-*Ser*, raised at the appropriate temperatures as above.

The construct UAS-*Nintra* was provided by M. Haenlin and is the same as that used by Klein and Martinez Arias (1998). This construct encodes a constitutively active, truncated form of N. UAS-*Nintra* expression was driven in the fourth and fifth tarsal segments by *apterous*-Gal4 (*ap*-Gal4) (FlyBase) using the following cross: *y w*; *ap*-Gal4 / CyO × UAS-*Nintra; Ser*^{RX106} / TM6b, pharate adults only appear at 18°C. The construct UAS-*ECN* (Extra Cellular Notch) encodes a dominant-negative form of N that maintains the extracellular ligand-binding and transmembrane domains, but lacks the intracellular domains required for signal transduction (Jacobsen et al., 1998). *Distalless*-Gal4 (*Dll*-Gal4) (Calleja et al, 1996) was used to drive the expression of UAS-*fng* (Kim et al., 1995) throughout the tarsal region using the following cross: *Dll*-Gal4 / CyO × UAS-*fng* /SM6a-TM6b /Ser^{RX106} *st e.*

Immunocytochemistry

Imaginal discs and pupae were staged according to their morphology. Third instar discs were fixed for 10 minutes in cold 4% paraformaldehyde in PBS, and pupal legs were fixed for 15 minutes. These were then stained, using standard procedures, for enzymatic X-gal staining, peroxidase or fluorescence.

Ser expression was detected with a mouse polyclonal anti-SER antibody (Speicher et al., 1994) diluted at 1:50 (fluorescence) or 1:1000 (peroxidase). After paraformaldehyde fixation, a further methanol fixation was carried out for 5 minutes at –20°C. Tween was used throughout the protocol instead of Triton X-100. *Dl* expression was detected using a mouse anti-DL antibody (Kooh et al., 1993) diluted at 1:10 (fluorescence), also with an additional methanol fixation. For fluorescent double stainings, anti-β-galactosidase raised in rabbit (Cappel) diluted at 1:5000 was used to detect *lacZ* reporter gene expression. *E(spl)mδ* and *mγ* expression was detected using the mouse monoclonal antibody mAb323 (Jennings et al., 1994). *N* expression was studied using the monoclonal antibody C17.9C6, which recognises the intracellular domain of the *N* protein (Fehon et al., 1991), at a concentration of 1:100 using the same protocol as for anti-SER and anti-DL antibodies. Secondary antibodies were from Vector and Jackson. X-gal and peroxidase images were photographed using a Leica microscope, and immunofluorescence images were captured on a Leica confocal microscope. Final figures were produced using scanned optical and confocal images assembled and processed with the Adobe Photoshop program.

RESULTS

It has been described that *N* and *Dl* are required for the correct segmentation of the legs in the fly (Parody and Muskavitch, 1993; Shellenbarger and Mohler, 1975) and that *N* is expressed in leg discs (Kidd et al., 1989). In order to ascertain the nature of these requirements, we decided to study the roles of *N*, *Ser* and *Dl* during leg development.

The development of joints and leg segments in *Drosophila*

The legs of *Drosophila* are jointed appendages, as in all other arthropod animals. In *Drosophila* and most insects, the structure of the legs is remarkably constant and is as follows. Every leg carries articulations, or joints, which divide the leg into parts, or segments. These segments are called, from proximal (or close to the body wall) to distal: coxa, trochanter, femur, tibia and five tarsal segments, plus a pre-tarsus, or claw organ, at the tip of the leg (Bryant, 1978). The joints differ from other parts of the leg in that they are devoid of bristles and include a flexible intersegmental membrane and interlocking parts composed of thickened cuticle. The details of the morphology of these parts varies from joint to joint and only the joints between the tarsal segments are identical and composed of a 'socket' in the proximal part of the joint and an interlocking 'ball' in the distal part (Fig. 1A) (Held et al., 1986). The other joints do not include a 'ball and socket' structure but a variety of condyles and cavities (Fig. 1B).

Joints are not obvious in the developing legs until the time of pupation, when the everting leg discs show a series of constrictions that different authors have identified with the presumptive positions of the final joints (Waddington, 1943; Fristrom and Fristrom, 1993). However, during subsequent metamorphosis, the legs inflate and lose these constrictions although different cell contours can still be seen at positions that seem to correlate with future joints (Fristrom and Fristrom, 1993). The lack of markers other than morphological ones has not allowed an exploration of joint development in more depth.

We have used a marker which seems to correlate faithfully with joints, an enhancer trap inserted into the *disconnected* (*disco*) gene (Heilig et al., 1991). The *disco* gene encodes a protein required for axonal migration and leg development (Heilig et al., 1991) and in the legs its expression is associated with joint development. *disco* expression is present in the developing leg imaginal disc at 120 hours after egg laying (Fig. 1C). Although it is expressed throughout the presumptive leg region, *disco* expression is upgraded in a series of rings around the centre of the disc. During the first 4 hours after puparium formation (APF), *disco* expression can be seen to become more strongly modulated in rings, which correspond with the presumptive leg segments (Fig. 1D). At around 12 hours APF, *lacZ* expression is restricted to these rings, which in a lateral view of the developing leg appear as stripes about 6 cells wide situated next to but proximal to constrictions in the presumptive tarsal region (Fig. 1E). Staining of adult flies carrying this marker shows *disco* expression specifically restricted to the joints (Fig. 1F).

Ser signalling controls the development of the leg joints

Null alleles of *Ser* are lethal but a few mutant flies are formed inside the pupal cases. These animals show a variety of developmental defects (Speicher et al., 1994; Couso et al., 1995), amongst them, leg deformities (Fig. 2C). The legs lack joints between all segments although sometimes a remnant constriction can still be seen. No other leg area is affected and the segment boundaries are still present, as shown by the apical bristles in tarsi and tibia. This mutant phenotype correlates with the pattern of expression of *Ser*. Using an antibody against

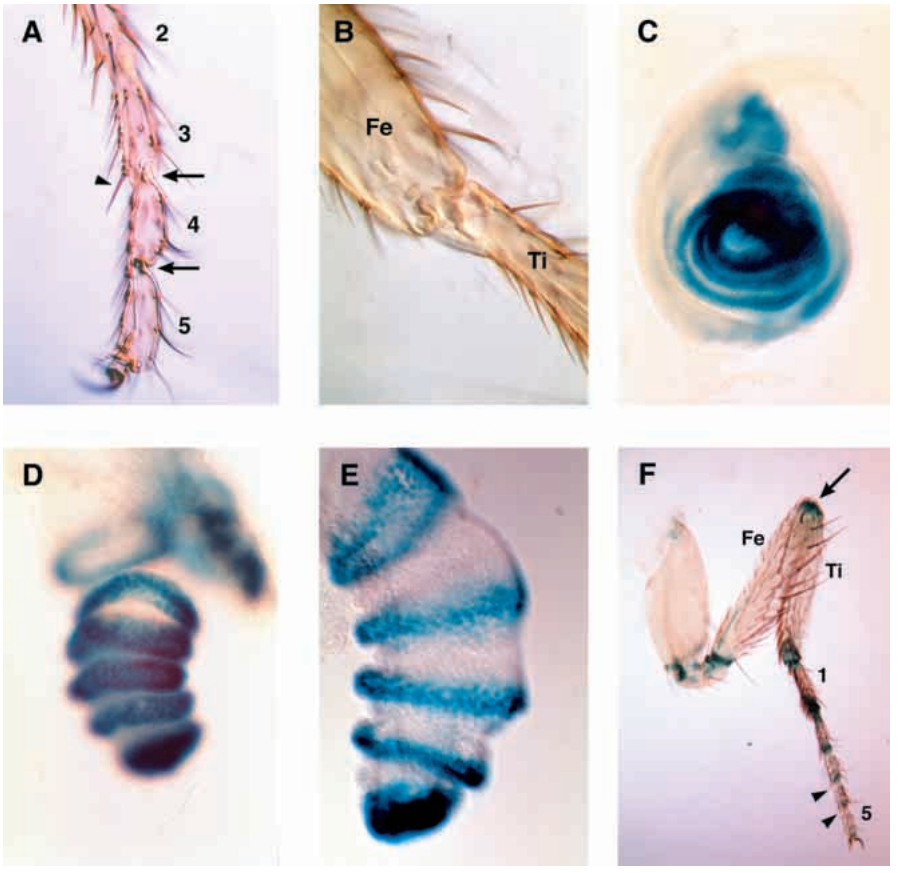

Fig. 1. Joint morphology and *disco* expression. (A) Detail of the tip of a wild-type leg, showing the tarsal joints (arrows). Proximal is at the top and distal to the bottom. 2-5, second to fifth tarsal segments; a ventral apical bristle marks the edge of each tarsal segment (arrowhead). (B) Wild-type joint between the femur (Fe) (top) and the tibia (Ti) (bottom). (C) Expression of the *disco-lacZ* reporter gene in a third instar leg imaginal disc. General expression over the presumptive leg region is seen, with upgraded concentric rings. (D) *disco* expression in a pupal leg 4 hours after puparium formation (4 hours APF) orientated as in A. *disco* expression is being refined into rings that encircle the developing leg. In this lateral view, the rings appear as stripes. (E) *disco* expression at 12 hours APF is restricted to stripes up to 8 cells wide. The stripes show a 'bell-shaped' distribution, with the 2-3 cells in the middle showing stronger expression. (F) *disco*-driven *lacZ* expression remains in the adult legs where it is restricted to the joints. Arrow, Fe-Ti joint; arrowheads point to the same tarsal joints (3/4 and 4/5) as the arrows in A. Fe, femur; Ti, tibia; 1,5, first and fifth tarsal segments.

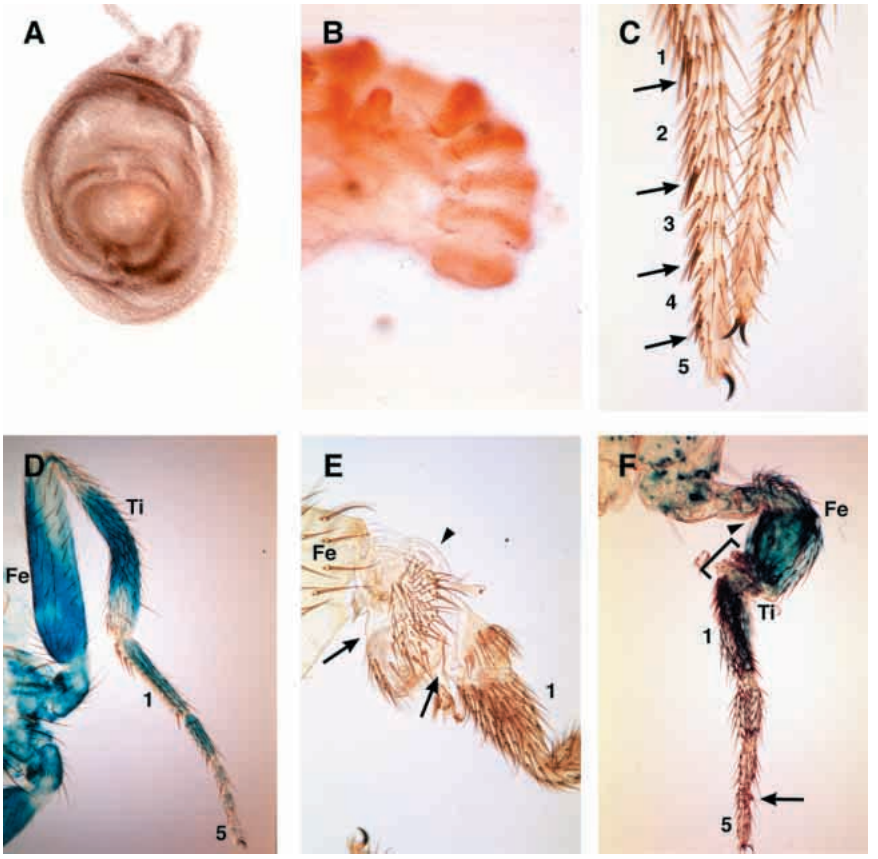

Fig. 2. *Ser* expression and function in legs. (A) Third instar leg imaginal disc stained with anti-SER antibody. *Ser* protein expression forms concentric rings. (B) *Ser* expression in stripes in a pupal leg 8 hours APF, revealed with an anti-SER antibody. The leg is curved so its proximal end is found to the left and its distal end points down. (C) Legs from a *Ser* null mutant animal. The joints are completely lost but other areas of the leg are not affected. Tarsal segments 1-5 are indicated and arrows mark the thick, dark apical bristles at the distal end of each segment where the joints are found in the wild type. (D) *lacZ* expression in the legs of a *klu*-Gal4 UAS-*lacZ* fly. *klu* is expressed in rings in the leg imaginal disc (not shown) and in the adult legs strong expression is seen outside the joint regions. Labels as in Fig. 1F. (E) Detail of the tibia from a *klu*-Gal4 UAS-*Ser* leg showing ectopic joint differentiation as constrictions (arrows), and intersegmental naked membrane (arrowhead). (F) Leg from a *disco-lacZ; klu-Gal4* UAS-*Ser* fly. Labels as in Fig. 1F. Ectopic *disco* expression is seen outside the joint areas where *disco* expression is restricted in the wild type (compare with Fig. 1F). Note the ectopic joints in the femur (arrrowhead), in the folded tibia (brackets) and in the fourth tarsal segment (arrow).

the *Ser* protein, expression is seen to appear in rings in third instar discs (Fig. 2A; Bachman and Knust, 1998) and later is found close to the presumptive joint areas in pupal legs. Stripes of *Ser* expression are seen proximal to constrictions in everting legs (Fig. 2B). Double staining with *disco* expression shows overlapping expression of *disco* and *Ser* (Fig. 3C-C″). However, in the 'bell-shaped' distribution of *disco*, cells with maximum levels of *disco* are located at the distal edge of the stripe of *Ser* expressing cells (Fig. 3D-D″).

These results show that *Ser* is required for the development of joints, and *Ser* expression adjacent to joint areas suggests that *Ser* could be directing joint development. If this were the case we would expect that ectopic expression of *Ser* would lead to the ectopic development of joints, and indeed we find this to be the case. We have used a *klu*-Gal4 line (Klein and Campos-Ortega, 1997), which is expressed in the legs outside the joints (Fig. 2D), to drive ectopic expression of a UAS-*Ser* construct. *klu*-driven expression of *Ser* leads to the ectopic development of joint-like cuticle, characterised by loss of bristles, cuticle thickenings and inpocketings (Fig. 2E). In leg regions like the tibia, where *klu* expression has defined boundaries, ectopic constrictions tend to appear. The transformation of interjoint leg regions towards joints is corroborated by the accompanying ectopic expression of *disco* (Fig. 2F). Reciprocally, in *Ser* mutants *disco* expression is lost after 12 hours APF (not shown).

The requirements for *Dl* are more extensive than for *Ser*

We have revealed *Dl* expression using a *Dl-lacZ* reporter allele and an antibody against the DL protein, and found *Dl* to be expressed throughout the presumptive leg at third instar, but with upgraded expression in rings (Fig. 4A). In pupal legs these rings can be seen to locate proximal to constrictions, and in adult legs *lacZ* expression is found at low levels throughout the leg, but it is stronger proximal to the joints (Fig. 4B). We have compared the expression of *Ser*, *disco* and *Dl* in pupal legs and found that the stripes of high *Dl* expression coincide with cells expressing *Ser* (Fig. 3A-A″, B-B″).

To study the requirements for *Dl* we have used a viable temperature-sensitive mutant combination of alleles that produces mutant leg phenotypes (Parody and Muskavitch, 1993). Following exposure to the restrictive temperature during the third instar and pupal periods when *Dl* is expressed near the presumptive joints, the *Dl* mutant legs are shortened. The tarsal segments are particularly reduced and have seemingly disappeared, but on close inspection it can be seen that the tarsal apical bristles are still present and sometimes some remnant joint structures as well (Fig. 4C). Because *Dl* and *Ser* are co-expressed, the joint defects in *Dl* mutants could be indirectly due to a loss of *Ser* expression in *Dl* mutants, but we examined this and found that the *Ser* stripes are still present in *Dl* mutant legs (Fig. 4D). Reciprocally, *Dl* expression is still present in *Ser* mutants (not shown), showing that the expression of *Ser* and *Dl* are not directly dependent on each other and that their mutant joint phenotypes reflect independent requirements. We interpret these results as showing that although joint areas require *Dl* for their development, the strongest requirement for *Dl* is in the regions located between segmental boundaries. This implies that the requirements for

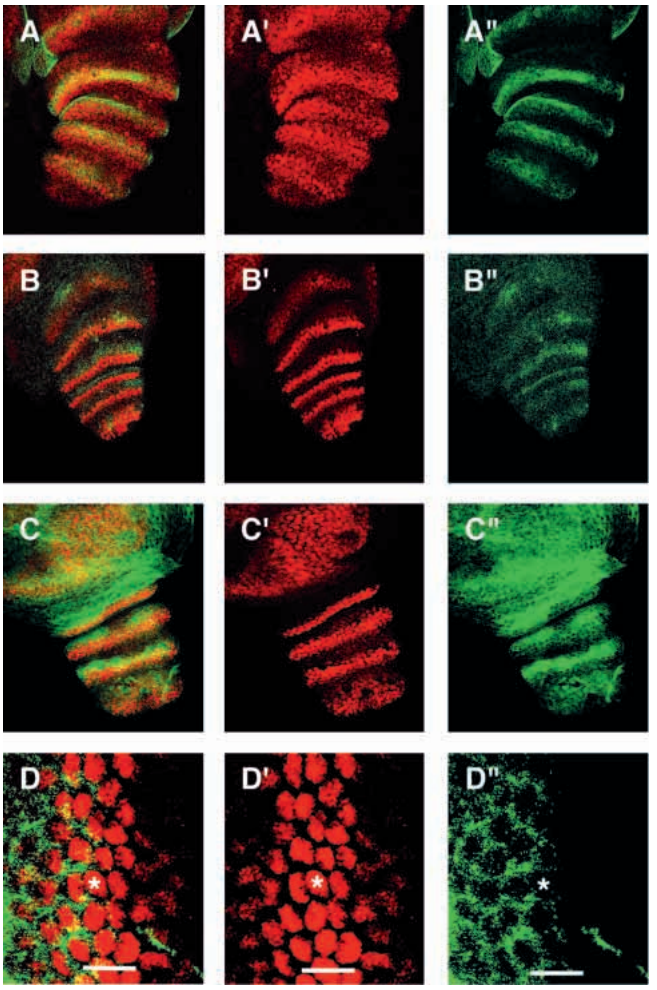

Fig. 3. Expression of *Ser*, *Dl* and *disco* in pupal legs. (A-A″) Confocal picture of a double antibody staining of a 4 hour APF pupal leg orientated as in Fig. 1D, showing expression of *Dl* (red; see also A′) and *Ser* (green; see also A″). *Dl* expression is revealed with an antibody against the β-gal protein expressed by the *Dl-lacZ* reporter gene, whereas *Ser* expression is revealed by anti-SER antibody. *Ser* expression is restricted to stripes whereas *Dl* is expressed at low levels throughout the leg with upgraded stripes that coincide with *Ser* expression. (B-B″) Expression of *Dl* and *disco* revealed with antibodies against the *Dl* protein (green; see also B″) and against the β-gal protein driven by *disco* (red; see also B′). *Dl* expression is upgraded proximal to *disco* stripes. (C-C″) Expression of *Ser* and *disco*, revealed with anti-SER antibody (green; see also C″) and anti-β-gal antibody (red; see also C′), respectively, in a fly carrying the *disco-lacZ* reporter gene. *Ser* expression is proximal to, but overlapping with, *disco*. (D-D″) High magnification detail of a leg stained as in C-C″. Proximal to the left, distal to the right. The bars mark the 2-3 cell wide stripe of cells with high levels of *disco* expression which straddles the edge of *Ser* expression. Cells with lower levels of *disco* expression can be seen at either side of this stripe. The asterisks label the same cell in these three panels.

Dl are more extensive than those of *Ser*, and this is corroborated by their different requirements for *disco* expression. In *Ser* mutant legs, the stripes of *disco* expression form but are not maintained properly. However, in *Dl* mutants, the stripes of *disco* are not formed correctly and instead wider and fewer rings remain (Fig. 4E,F), a pattern similar to that of *Ser* expression in *Dl* mutants. We interpret this *disco* expression in *Dl* mutants as a corroboration of the main requirement for *Dl* being in the intersegmental regions. Failure of these regions to develop causes the absence of non-*disco* expressing cells between *disco* stripes so that fewer but wider *disco* stripes appear in the mutants.

The differences between the requirements and effects of *Ser* and *Dl* are again shown by comparison of their ectopic expression. *klu*-Gal4 UAS-*Dl* produces no visible phenotype in the interjoint regions of the femur and the tibia, but it expands the area differentiating cuticular joint characteristics, like naked and thick cuticle, in the femoral, tibial and tarsal joints (Fig. 4G). *klu*-driven *Dl* expression produces no phenotype in the interjoint regions, presumably because *Dl*, unlike *Ser*, is already expressed there and therefore *klu*-driven *Dl* expression is not ectopic. Effects are only seen near *Ser* expressing areas, presumably because a wider co-expression region is created. In the tarsal regions in addition to this joint-extension phenotype a tarsal loss is seen (Fig. 4H) following *Dl* expression driven by either *klu* or *ap*-Gal4 (which drives expression in the presumptive regions of forth and fifth tarsal segments during third instar and pupa). This tarsal reduction does not result from the formation of ectopic structures and thus it is likely to be caused by a negative effect of *Dl*, since it mimics a *Dl* or *N* lack of function phenotype (see also below).

In spite of the differences in the requirements for *Dl* and *Ser*, there is an overlap in that they are both required for the development of joints. It is possible that this overlap explains why the requirements of *Dl* in the joints are weaker than those in the interjoints. We decided to study whether this overlapping requirement is mediated by *N*.

The requirements for *N* are a composition of the requirements for *Ser* and *Dl*

In spite of their different but partially overlapping requirements, *Ser* and *Dl* signalling are thought to be mediated by the same receptor, N, which is expressed ubiquituously (Kidd et al., 1989; Kooh et al., 1993). We studied the requirements for *N* function in leg development using different temperature-sensitive mutant combinations of alleles that produce adult flies (Shellenbarger and Mohler, 1975; Couso and Martinez Arias, 1994). When exposed to the restrictive temperature during third instar and pupal phases, *N* mutant flies show a marked reduction in leg length with all areas of the leg segments being affected (Fig. 5A,B). Joints are completely lost but also often apical bristles. The overall length of the segments, and especially of the tarsal region, is more reduced than in *Ser* or *Dl* mutants because both joint and interjoint tissue is missing (Fig. 5B). Thus, the *N* mutant phenotype looks like a composition of the *Ser* and *Dl* mutant phenotypes. When the expression of *disco* is revealed in *N* mutants, a combination of *Ser* and *Dl* phenotypes is also seen. *disco* stripes do not resolve properly, as in *Dl* mutants, and then they are subsequently lost, as in *Ser* ones (not shown). To analyse separately the requirements for *N* in the interjoint regions, we have expressed a dominant-negative form of N (UAS-ECN) in the interjoint regions by using *klu*-Gal4. In *klu*-Gal4 UAS-*ECN* flies, the legs are shortened due to the loss of interjoint tissue but the joints are still present and sometimes fused (Fig. 5C).

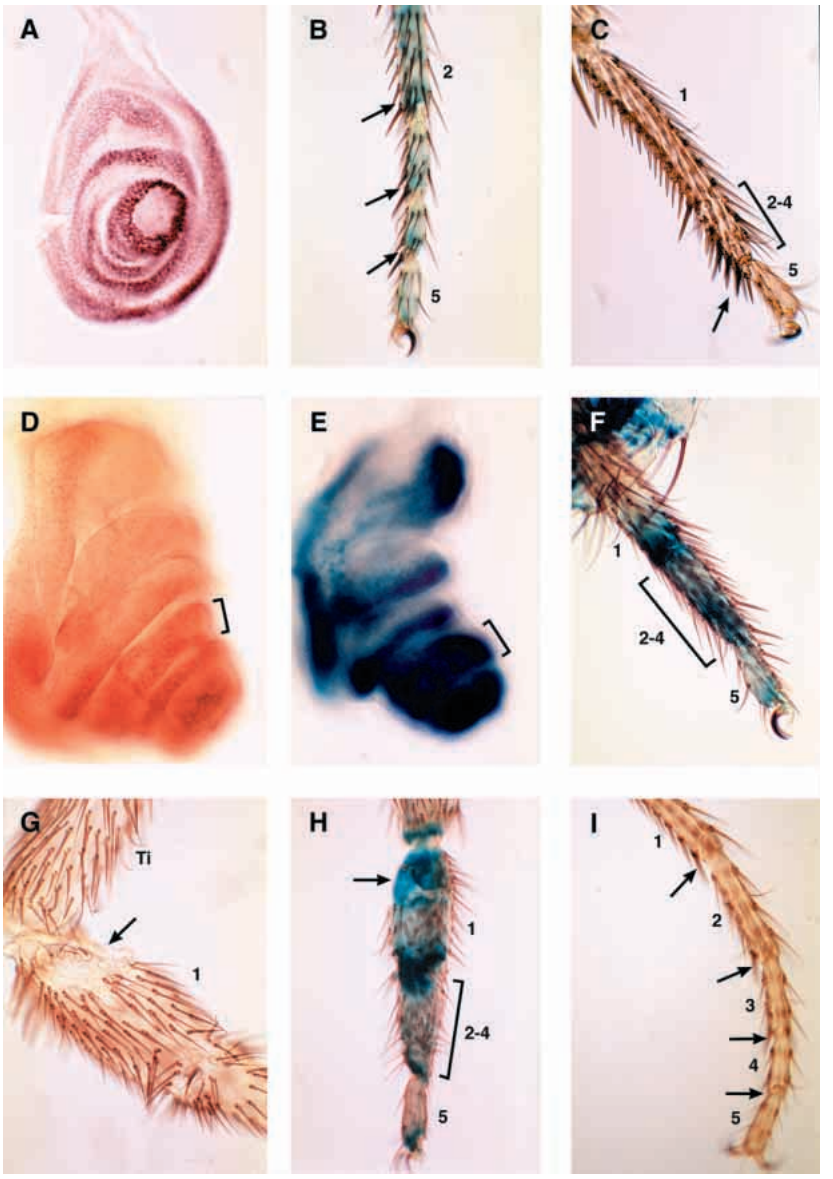

Fig. 4. *Dl* expression and function in the legs. (A) *Dl*-driven *lacZ* expression in rings in a leg imaginal disc just before pupariation, revealed with an anti-β-gal antibody. (B) Detail of the second to fifth tarsi from an adult fly showing *Dl*-driven *lacZ* expression. Low expression is seen throughout the leg and upgraded at the proximal side of the joints (arrows). (C) Tarsal region from the adult leg of a *Dl* temperature-sensitive mutant exposed to the restrictive temperature from the third larval instar until the end of development. Tarsal segments are reduced (remnants of segments 2-4 are indicated by a bracket), and in some cases only the thick, black apical bristles remain (arrow). Joints are reduced as well although in some cases they are not completely lost (see edges of bracket and compare with wild-type legs in Figs 1A and 4I, and *Ser* mutant legs in Fig. 2C). (D) *Ser* expression revealed with an anti-SER antibody is still present in a pupal leg from a *Dl* mutant, sibling of C. The bracket indicates two fused SER stripes. (E) *disco* expression in a *Dl* mutant as in C and D. Instead of five wild-type tarsal stripes (see Fig. 1D) only three are seen; the bracket indicates a wider stripe, which results from the fusion of two or more *disco* stripes. (F) Adult leg of a sibling of E. Fused tarsal segments 2-4 are indicated by the bracket. (G) Detail of a leg from a *klu*-Gal4 UAS-*Dl* animal. An expansion of the tibia/first tarsus joint region is apparent in that there is an extended region of naked, thickened cuticle (arrow). (H) Leg from a *disco-lacZ*; *klu*-Gal4 UAS-*Dl* fly. Tarsal segments 2-4 are reduced (brackets), while joint regions around tarsus 1 are expanded, as seen by the expansion of both joint cuticle (arrow) and *disco* expression (compare with Figs 1F, 4G and 4I). (I) Tarsal segments (1-5) from a wild-type leg. Joints are marked by arrows.

These leg phenotypes thus resemble those produced in interjoint regions by loss of *Dl* function (see Fig. 4C-F).

Expression of a truncated and constitutively activated form of N (UAS-*Nintra*) driven by *klu*-Gal4 is lethal. Using an *ap*-Gal4 driver it can be seen that the fourth tarsal segment becomes hyper-jointed in that double ball joints are formed, similar to the effects of UAS-*Ser* driven by *ap*-Gal4 or *klu*-Gal4 (Fig. 5D). In addition, the interjoint region is reduced, either as a consequence of its conversion to extra joint tissue, or to an inability to develop the interjoint cell fates which have low, but not high, levels of N activation. These tarsal phenotypes are thus different from those seen with UAS-*Dl* and UAS-*ECN*, because UAS-*Nintra* and UAS-*Ser* do not involve the loss of entire tarsal segments.

Altogether these results suggest that the overlap of *Ser* and *Dl* expression and requirements at the joints is mediated by N activation. As a marker of N activity, we have monitored the expression of members of the *E(spl)* complex (Jennings et al., 1994). Using reporter constructs with the regulatory regions of *E(spl)* (Kramatschek and Campos-Ortega, 1994), which reproduce the endogenous *E(spl)* expression in the leg discs (de Celis et al., 1996), it can be seen that *E(spl)m8* expression is related to joints while *m5* and presumably *m6* are not. Expression of the *E(spl)m8* reporter construct in third instar discs is initially strong in regions undergoing PNS development. In the legs, these correspond to the chordotonal organs in the femur and the tibia (see also Jarman and Ahmed, 1998). In addition, expression near the presumptive joints is seen to appear, and then resolve in the pupa into one-cell wide stripes proximal to the leg constrictions (Fig. 5E), in positions that correlate with cells with maximum levels of *disco* expression. A similar although much weaker pattern of expression of *E(spl)mδ* is seen as revealed by the mAb323 antibody (Jennings et al., 1994) (not shown). Another marker of N activity is the expression of *N* itself, which becomes upregulated in cells where *N* signalling is being received (de Celis et al., 1997). Using an anti-N antibody, we observe upregulated expression of N immediately proximal to the

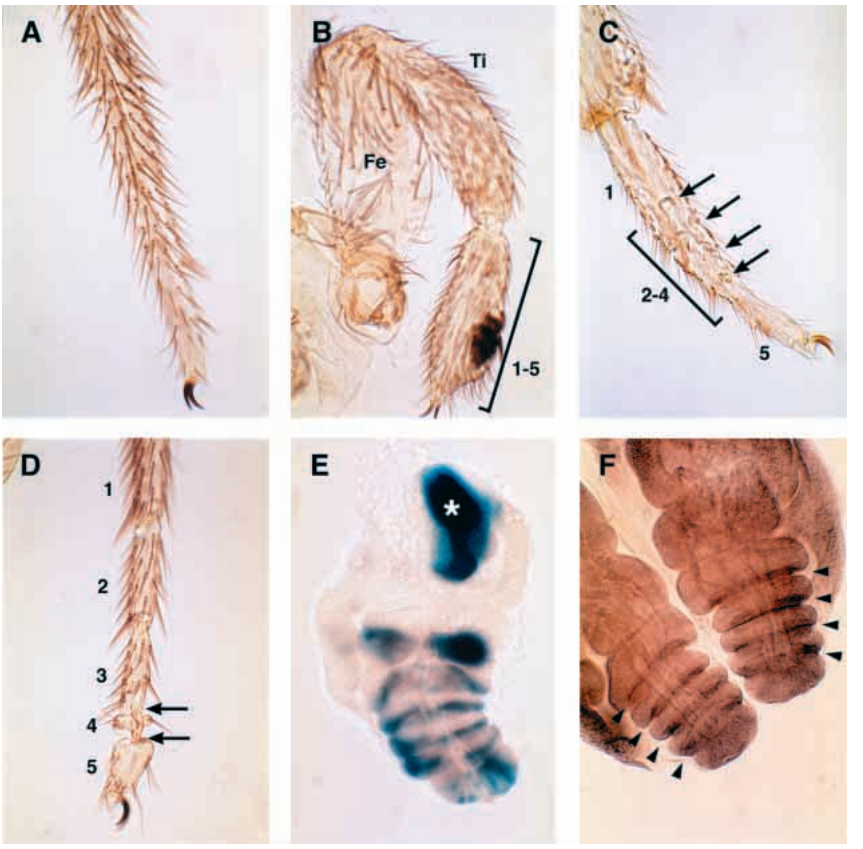

Fig. 5. *N* function in *Drosophila* legs. (A) Legs from a *N* mutant animal with partial loss of *N* function (N^{55e11}/N^{ts} raised at 17°C). Note the lack of joints and apical bristles. (B) Legs resulting from an almost total loss of *N* function (N^{55e11}/N^{ts} exposed to 25°C during the third larval instar and pupal periods), showing loss of tarsal segments (bracket). (C) Leg from a *klu*-Gal4 UAS-*ECN* fly. Expression of the dominant-negative N protein ECN driven by *klu*-Gal4 in the interjoint regions produces loss of interjoint tissue. The joints are still present (arrows) but appear fused in some cases. Interjoint loss and joint fusion resemble those produced in *Dl* mutants (see Fig. 4C-F). (D) Distal end of the leg from an *ap*-Gal4 UAS-*Nintra* fly. Double ball joints with reversed polarity are formed in tarsus 4 (arrows). (E) *E(spl)m8* expression as revealed by the *E(spl)m8 2.61-lacZ* reporter construct in a leg 4-8 hours APF. Expression is seen in one-cell wide stripes at the proximal side of the constrictions. Asterisk, chordotonal organ. (F) *N* expression revealed by an antibody against the intracellular domain of N. N expression accumulates in cells immediately proximal to the constrictions (arrowheads).

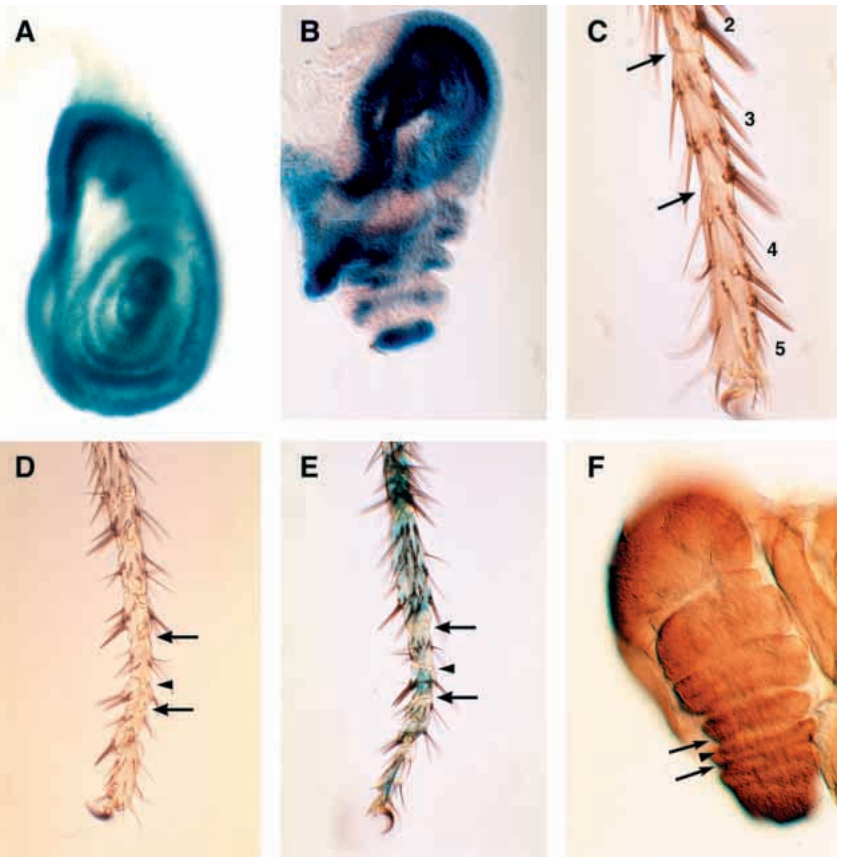

Fig. 6. Repression of joint development by *fng* and *dsh*. (A) *fng* expression, revealed by a *lacZ* reporter gene, is seen in concentric rings in leg discs just before pupariation. (B) Stripes of *fng* expression (revealed as in Fig. 6A) in a 4 hour APF pupal leg. (C) Ectopic expression of *fng* throughout the whole tarsal region in *Dll*-Gal4 UAS-*fng* animals only produces a mutant phenotype at the joints, which are reduced (arrows) or completely eliminated (see 4/5 tarsal joint). (D) Tarsi of a *dsh* mutant adult leg. Ectopic joints with reversed orientation of the 'ball and socket' appear proximal to the normal joints. The endogenous joints at both ends of tarsal segment 3 are marked with arrows and the ectopic joint in this tarsal segment is marked by an arrowhead. (E) *Dl*-driven *lacZ* staining in the tarsi of a *dsh* mutant leg. *Dl* expression is normal (compare with Fig. 4B) but ectopic joints form proximal to the stripe of high *Dl* expression (arrowhead). Arrows and arrowhead as in D. (F) *N* expression revealed by anti-N staining in a *dsh* mutant pupal leg. Proximal to the endogenous stripes of upregulated N expression, ectopic stripes can be seen. Arrows and arrowhead as in D and E.

constrictions in pupal legs (Fig. 5F). At high magnification it can be appreciated that this upregulation is restricted to a single row of cells at this position (not shown), thus confirming that SER and DL are triggering *N* signalling in these cells.

Other elements in *N* signalling and allocation of joints

The results presented suggest a model in which the co-expression of *Ser* and high levels of *Dl* in a stripe of cells proximal to the future presumptive joints activate N in cells adjacent but distal to this stripe. We wondered whether this specificity could be due to the presence of other factors that would be interfering with *Ser* and *Dl* signalling in cells located inside the *Ser-Dl* stripe or proximal to it. In the DV boundary of the wing, the membrane protein encoded by the gene *fringe (fng)* has been postulated to modulate *N* signalling by interfering with *Ser* signalling (Fleming et al., 1997a; Panin et al., 1997). In the developing legs, *fng* is expressed in stripes or rings around the positions of presumptive joints (Fig. 6A,B). Using a UAS-*fng* construct (Kim et al., 1995), we misexpressed *fng*. Ectopic expression of *fng* outside the joints driven by *klu*-Gal4 produces no phenotype. However, uniform tibial and tarsal *fng* expression driven by *Dll*-Gal4 only affects the joints, which are reduced or disappear, a phenotype reminiscent of that of *Ser* mutants (Fig. 6C). Thus, *fng* activity in the leg seems to be restricted to a repression of joint development around presumptive joint areas. It is possible that *fng* expression in the wild type is repressing *N* signalling in cells located in the *Ser-Dl* stripe or proximal to it, providing the polarity in the joint-promoting function of *Ser* and *Dl*. Consistent with this proposal, we also observe in the wings of UAS-*fng* flies margin nicks and thickening of veins, phenotypes characteristic of loss of *N* signalling in these developmental processes (Couso et al., 1995; de Celis et al., 1997). Our results also suggest a possible role for other factors in the definition of the polarity of *Ser* and *Dl* signalling. *dishevelled* (*dsh*) mutant legs develop ectopic joints (Held et al., 1986) (Fig. 6D). The expression of *Dl* is normal in *dsh* mutant legs and it can be seen that the ectopic joints appear proximal to the cells expressing high levels of *Dl* (Fig. 6E). This ectopic joint differentiation in *dsh* mutant legs is preceeded by ectopic *N* activation, as revealed by staining with anti-N antibody (Fig. 6F). These results are compatible with a role for *dsh* in the repression of *N* signalling and joint development in cells proximal to the *Ser* and *Dl* stripe.

DISCUSSION

A combination of signals from *Ser* and *Dl* establishes leg segments and joints

Our results suggest a model in which the co-expression of *Ser* and high levels of *Dl* in a stripe of cells activate N in cells adjacent but distal to this stripe. Activation of N promotes expression of members of the *E(spl)* complex and leads to joint formation and *disco* expression (Fig. 7A).

The only function of *Ser* in leg development is to promote joint formation, a conclusion supported by the recent findings of de Celis et al. (1998), which are similar to our own. Complete loss of *Ser* eliminates joints and *disco* expression, whereas ectopic *Ser* produces ectopic joints and ectopic *disco* expression. However, we observe that *Dl* seems to have more than a simple joint-promoting role in leg development.

Loss of *Dl* eliminates first the regions between *disco* /*Ser*-expressing rings, but also, secondly, joints. Since loss of interjoint regions is also seen both in *N* mutants and following expression of a dominant-negative form of *N*, we postulate that *Dl* expression in the interjoint regions produces low levels of activation of N that do not lead to *E(spl)* expression but which allow cell survival and/or cell proliferation. A requirement for

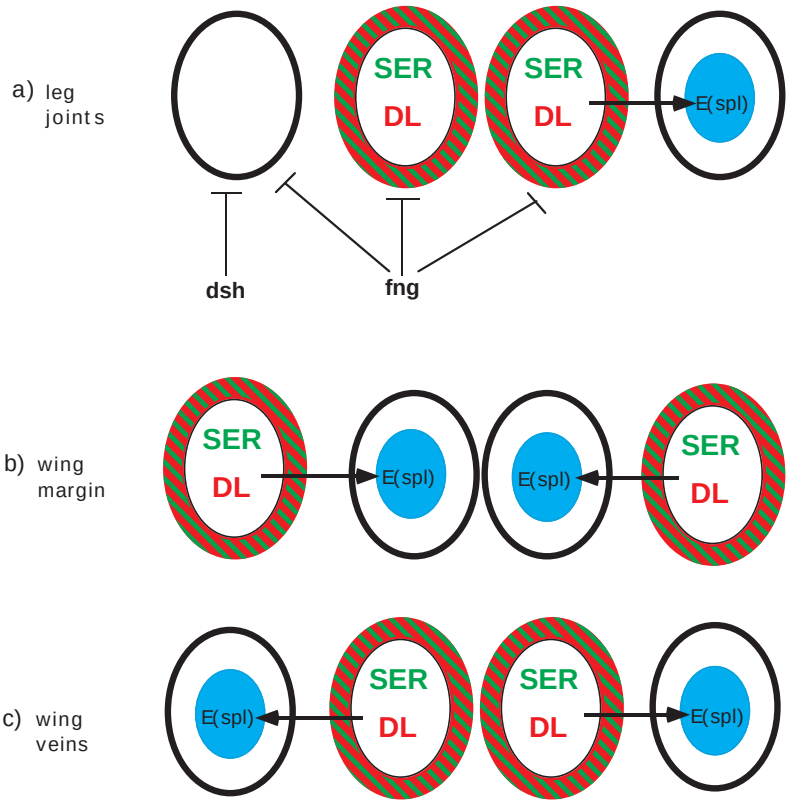

Fig. 7. Models for *N* signalling-mediated boundary formation. (A) *N* signalling in the leg joints. Co-expression of SER and high levels of DL near the distal end of each leg segment leads to *E(spl)* expression and joint development in adjacent distal cells (to the right). *fng* and *dsh* activity repress *N* signalling to cells proximal to the *Ser* and *Dl* stripe, whereas a combination of *fng* and autonomous dominant negative effects of SER and DL represses signalling in *Ser* and *Dl* expressing cells. (B) *N* signalling in the wing margin during late third instar. Coincident and parallel double stripes of *Ser* and *Dl* expressing cells signal to wing margin edge cells located in between them (Couso et al., 1995; Kim et al., 1995; de Celis and Bray, 1997; Micchelli et al., 1997). Repression of signalling in *Ser* and *Dl* expressing cells is attributed to autonomous negative effects of SER and DL (Michelli et al., 1997). (C) *N* signalling in the wing veins. Stripes of cells with vein fate express *Ser* and *Dl*, whose signalling to cells at both sides of the stripe inhibits them from becoming veins (de Celis et al., 1997; Zeng et al., 1998). It is proposed that autonomous signalling is avoided by downregulation of *N* expression within the *Ser* and *Dl* stripes (de Celis et al., 1997).

N in cell growth or survival has been noted in the wing blade where this and other effects of *N* signalling seem to be mediated by unknown factors other than *E(spl)* (de Celis et al., 1996). The requirement for *Dl* in joint development is not mediated by *Ser* because *Ser* and *Dl* expression are not directly dependent on each other. Joint loss in *Dl* mutants is presumably less severe than interjoint loss because *Ser* and *Dl* expression could be synergistic and partially redundant (Micchelli et al., 1997; Zeng et al., 1998). The combined and potentially synergistic effects of *Ser* and *Dl* would produce a high level of activation of N that would lead to expression of members of the *E(spl)* complex, upregulation of *N* expression, and to joint development and *disco* expression. Thus, we believe that combinations of signalling by *Ser* and *Dl* could produce different levels of activation of N, which in turn are translated into different downstream effects. As noted in other systems (de Celis et al., 1996) these downstream effects of *N* signalling should be mediated by more factors than just *E(spl)*, since *E(spl)* mutant legs have been reported as having a wild-type phenotype (de Celis et al., 1998).

The width of the final joint region is wider than the single row of cells activated by the membrane-tethered SER and DL proteins and visualised by *E(spl)* expression. In principle it is possible that the cells of the whole final joint all descend from the *E(spl)* expressing cells, but previous studies have shown that only one or two cell divisions occur in the legs after puparium formation (Graves and Schubiger, 1982). Thus it is likely that in the *E(spl)* expressing cells another cell signalling molecule is activated, which in a secondary event would define a wider joint presumptive region, just as *N*-induced expression of the secreted signalling *wingless* protein defines the presumptive wing margin (Couso et al., 1994; Micchelli et al., 1997). A reflection of this putative second signalling event in the joints can be seen in the expression of *disco*. *disco* expression is dependent on *Ser* but it is wider than the single row of cells where N is activated and thus it cannot be directly reflecting *N* signalling at the joint. However, the 'bell-shaped' distribution of *disco* might reflect this putative secondary signalling event, with a maximum in cells at the edge of the *Ser-Dl* stripe. The nature of the joint-promoting putative secondary signal is unknown at the moment, but one possible component is the product of the *four-jointed (fj)* gene. The *fj* protein is a putative signalling molecule that is expressed and required at the joints (Villano and Katz, 1995). *fj* expression has recently been shown to depend on *fng* and *N* signalling during eye development (Papayannopoulos et al., 1998), and it is lost in *N* mutant legs (S. A. Bishop and J. P. Couso, unpublished observations).

Further elements in joint development

An aspect of joint development which is not explained by the model presented is the polarity with which the stripe of *Ser* and *Dl* signals. Lack of signalling in wild-type *Ser* and *Dl* expressing cells could be due to an autonomous dominant negative effect of these molecules. Such an effect has been postulated before following ectopic expression experiments (Doherty et al., 1996; Jonsson and Knust, 1996; de Celis and Bray, 1997; Klein et al., 1997), and it has been shown to be present in the wing margin in lack of function conditions (Micchelli et al., 1997). Although an interaction of N and DL proteins in the same cell has been recently shown (Jacobsen et al., 1998), the molecular basis of such a negative effect remains unexplained. We find negative effects (tarsal segment loss) following overexpression of DL driven by *klu* or *ap*-Gal4, and so a putative dominant negative effect could be at work in the *Ser* and *Dl* stripes and explain the lack of signalling there. Similar dominant-negative effects of DL and also SER have also been reported by de Celis et al. (1998). However, an autonomous negative effect does not explain why cells adjacent but proximal to the *Ser-Dl* stripe do not seem to be signalled either.

A possible explanation would be either an asymmetric distribution of SER and DL, forming gradients like those seen in the late third instar wing margin (Micchelli et al., 1997) and in ectopic expression situations (Doherty et al., 1996; Kim et al., 1995), or a downregulation of *N* expression as has been noted in the developing wing veins (de Celis et al., 1997). The SER and DL stripes in legs show no apparent asymmetry but N distribution, although ubiquitous and initially uniform (Kidd et al., 1989), becomes upregulated in cells distal to the *Ser-Dl* stripes. Low availability of N protein could have an effect on the intensity of *N* signalling, but since upregulation of N is in itself a consequence of *N* signalling (de Celis et al., 1997), some other factor must polarise the signalling initially. Another explanation would rely on the action of a repressor acting upon cells proximal to the stripe. The phenotypes obtained after ectopic expression of *fng* are consistent with such a role for *fng*, as postulated in the wing (Fleming et al., 1997a; Panin et al., 1997). The expression of *fng* in the leg, which has been described as complementary to that of *E(spl)*, that is, present in non-signalled cells but excluded from joint forming ones (de Celis et al., 1998), is also consistent with this hypothesis. Such a function of *fng* could also repress *Ser* and *Dl* signalling in the stripe without recourse, or in addition, to putative autonomous dominant negative effects of SER and DL (Klein and Martínez Arias, 1998; Jacobsen et al., 1998). However, other factors could also be involved, such as the cell polarity pathway (reviewed in Gubb, 1998). Mutant phenotypes for *dsh* and other members of the cell polarity pathway produce ectopic joints with reversed polarity (Held et al., 1986), which we find to appear just proximal to the position of *Ser* and *Dl* stripes. Furthermore in *dsh* mutants we observe ectopic N activation proximal to the *Ser-Dl* stripe. Since the *dsh* protein has been shown to interact with N, and *dsh* has been postulated to inhibit *N* signalling in this manner (Axelrod et al., 1996), the cell polarity pathway could be involved in repressing *Ser* and *Dl* signalling to cells proximal to the *Ser* and *Dl* stripe (Fig. 7A).

Quantitative and qualitative effects of N ligands

The SER and DL ligands seem to produce the same effects on N, and only a synergistic but quantitative effect has been found in most processes examined (Gu et al., 1995; Micchelli et al., 1997; Klein and Martinez Arias, 1998; Zeng et al., 1998). Why then are there two N ligands in *Drosophila*? This could be just a device to increase the amount of *N* signalling beyond what is achievable by regulating the expression of a single ligand. It might not be possible to either completely eliminate or greatly imbalance *Dl* expression in certain areas without compromising the process of neurogenesis (Heitzler and Simpson, 1991). The addition of SER, a molecule mostly not involved in neural precursor selection (Gu et al., 1995), might

provide for further extremes of *N* signalling, which could also include dominant-negative effects. This greater quantitative range in the amount of *N* signalling when induced by the combination of SER and DL (ranging from high to moderate and to low or no signal) could then trespass certain thresholds and be converted into different qualitative gene regulation effects. In these terms a situation with a single ligand, DL, involved in neurogenesis could be the ancestral one, while the evolution of a second ligand, SER, would allow the co-option of the *N* signalling pathway to other pattern forming processes.

Topological aspects of boundary making by *N* signalling

Ser, *Dl* and *N* signalling is used in *Drosophila* to create boundaries in leg joints, the wing margin, and in the wing veins. In all these cases, the common link is the creation of boundaries between cell populations, sometimes to allow the singling out of boundary cells with new signalling properties. In vertebrates, homologues of *Ser*, *Dl* and *fng* are involved in similar processes, as in somite and rhombomere development (Conlon et al., 1995; Hrabe de Angelis et al., 1997; Cohen et al., 1997). In these varied developmental processes, and in the scenario of SER and DL having overlapping and similar effects on N, the precise outcomes of their signalling seem to stem from the different deployments of *Ser*, *Dl*, *N* and *fng* expression (Fig. 7). In leg joint development coincident stripes of cells expressing *Ser* and *Dl* signal not to themselves, but to cells adjacent to only one side of them (Fig. 7A). Autonomous *Ser-Dl* signalling seems to be blocked by *fng*, although a dominant negative effect by SER and DL cannot be excluded. Restriction of non-autonomous signalling to one side of the *Ser-Dl* stripe seems due to a combination of repression by *fng* and *dsh* plus a modulation of *N* expression. This situation is similar to somite boundaries in vertebrates where *Ser*, *Dl* and *fng* homologues are co-expressed and help to maintain an adjacent somite boundary through *N*. Alterations of *fng*, *Dl* or *N* function result in somite fusion and loss (Conlon et al., 1995; Hrabe de Angelis et al., 1997; Cohen et al., 1997), phenotypes which are reminiscent of the tarsal segment mutant phenotypes presented here.

During *Drosophila* wing margin development the situation is similar to joint development, with coincident patterns of *Ser* and *Dl* expression signalling to a boundary of adjacent cells (Fig. 7B). *Ser* and *Dl* expressing cells do not receive any signalling due to autonomous dominant negative effects (Micchelli et al., 1997). In the developing wing veins during pupal development, autonomous signalling seems to be avoided by exclusion of *N* expression in the vein stripes (de Celis et al., 1997). Thus a common outcome, the signalling of an adjacent row of cells, is apparently achieved by different mechanisms in the joints, the wing margin and the veins. However, in the wing margin (de Celis and Bray, 1997) and joints, expression of *N* seems to be regulated as in the veins, being low in *Ser-Dl* stripes but higher in the adjacent signalled cells. Moreover, *fng* is expressed near the presumptive wing margin and veins (Irvine and Wieschaus, 1994; S. A. Bishop and J. P. Couso, unpublished observations), where it might be repressing *N* signalling since overexpression of *fng* produces *N*-like phenotypes in these places.

N signalling would appear then to be a kit that can be used in different topological and molecular combinations of signalling elements. A general boundary-making activity of *N* signalling could entail definition of boundaries of cells by: (1) expression of ligands in cells close to the future boundary, (2) activation of N in adjacent cells at the boundary, and (3) repression of signalling in ligand expressing cells (and in some other cells if required) by a combination of ligand dominant negative effects, inhibition by *fng* and other repressors, and downregulation of *N* expression.

We thank N. Sommerville and S. Curran for technical assistance, E. Knust for providing flies and results prior to publication and anti-SER antibody, and S. Bray for mAb323 antibody. We thank D. Hartley, S. Greig and I. Galindo for constructive criticism. This research has been funded by The Wellcome Trust.

REFERENCES

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S. and Perrimon, N. (1996). Interaction between *wingless* and *Notch* signalling pathways mediated by *dishevelled*. *Science* **271**, 1826-1832.

Axelrod. J. D., Miller, J. R., Shulman, J. M., Moon, R. T. and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. *Genes Dev.* **12**, 2610-2622.

Bachmann, A. and Knust, E. (1998). Dissection of cis-regulatory elements of the *Drosophila* gene *Serrate*. *Dev. Genes Evol.* **208**, 346-351.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Bryant, P. (1978). Pattern formation in imaginal discs. In *The Genetics and Biology of* Drosophila (ed. M. Ashburner and T. Wright), pp. 230-335. Academic Press, London, New York, San Francisco.

Calleja, M., Moreno, E., Pelaz, S. and Morata, G. (1996). Visualization of gene expression in living adult *Drosophila. Science* **274,** 252-255.

Cohen, B. et al. (1997). Fringe boundaries coincide with Notch-dependent patterning centres in mammals and alter Notch-dependent development in *Drosophila*. *Nat. Genet.* **16,** 283-288.

Conlon R. A., Reaume, A. G. and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. *Development* **121**, 1533-1545.

Couso, J. P. and Bishop, S. A. (1998). Proximo-distal development in the legs of *Drosophila*. *Int. J. Dev. Biol.* **42**, 345-352.

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

Couso, J. P., Knust, E. and Martinez Arias, A. (1995). *Serrate* and *wingless* cooperate to induce *vestigial* gene expression and wing formation in *Drosophila*. *Curr. Biol.* **5**, 1437-1448.

Couso, J. P. and Martinez Arias, A. (1994). *Notch* is required for *wingless* signalling in the epidermis of *Drosophila*. *Cell* **79**, 259-272.

de Celis, J. and Bray, S. (1997). Feed-back mechanisms affecting *Notch* activation at the dorsoventral boundary in the *Drosophila* wing. *Development* **124**, 3241-3251.

de Celis, J. F., Bray, S. and Garcia-Bellido, A. (1997). *Notch* signalling regulates *veinlet* expression and establishes boundaries between veins and interveins in the *Drosophila* wing. *Development* **124,** 1919-1928.

de Celis, J. F., de Celis, J., Ligoxygakis, P., Preiss, A., Delidakis, C. and Bray, S. (1996). Functional relationships between *Notch*, *Su(H)* and the bHLH genes of the *E(spl)* complex: the *E(spl)* genes mediate only a subset of *Notch* activities during imaginal development. *Development* **122**, 2719-2728

de Celis, J. F., Tyler, D. M., de Celis, J. and Bray, S. (1998). Notch mediates segmentation of the *Drosophila* leg. *Development* **125**, 4617-4626

Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L. and Jan, Y. (1996). *Delta* is a ventral to dorsal signal complementary to *Serrate*, another *Notch* ligand, in *Drosophila* wing formation. *Genes Dev.* **10**, 421-434.

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

Fleming, R. J., Gu, Y. and Hukriede, N. A. (1997a). *Serrate*-mediated

activation of *Notch* is specifically blocked by the product of the gene *fringe* in the dorsal compartment of the *Drosophila* wing imaginal disc. *Development* **124**, 2973-2981.

Fleming, R. J., Purcell, K. and Artavanis-Tsakonas, S. (1997b). The *Notch* receptor and its ligands. *Trends Cell Biol.* **7**, 437-441.

Fristrom, D. and Fristrom, J. W. (1993). The metamorphic development of the adult epidermis. In *The Development of* Drosophila melanogaster, vol. 2 (ed. M. Bate and A. Martinez Arias), pp. 843-898. Cold Spring Harbour Laboratory Press, New York.

Graves, B. J. and Schubiger, G. (1982). Cell cycle changes during growth and differentiation of imaginal leg discs in *Drosophila melanogaster*. *Dev. Biol.* **93**, 104-110.

Gu, Y., Hukriede, N. A. and Fleming, R. J. (1995). *Serrate* expression can functionally replace *Delta* activity during neuroblast segregation in the *Drosophila* embryo. *Development* **121**, 855-865.

Gubb, D. (1998). Cellular polarity, mitotic synchrony and axes of symmetry during growth. Where does the information come from? *Int. J. Dev. Biol.* **42**, 369-377.

Heilig, J. S., Freeman, M., Laverty, T., Lee, K. J., Campos, A. R., Rubin, G. M. and Steller, H. (1991). Isolation and characterization of the *disconnected* gene of *Drosophila melanogaster*. *EMBO J.* **10**, 809-815.

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

Held, L., Duarte, C. and Derakhshanian, K. (1986). Extra tarsal joints and abnormal cuticular polarities in various mutants of *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **195**, 145-157.

Hrabe de Angelis, M., McIntyre II, J. and Gossler, A. (1997). Maintenance of somite borders in mice requires the *Delta* homologue. *Nature* **386**, 717-721.

Irvine, K. and Wieschaus, E. (1994). *fringe*, a boundary-specific signalling molecule, mediates interactions between dorsal and ventral cells during *Drosophila* wing development. *Cell* **79**, 595-606.

Jacobsen, T., Brennan, K., Martinez-Arias, A. and Muskavitch, M. (1998). Cis-interactions between *Delta* and *Notch* modulate neurogenic signalling in *Drosophila*. *Development* **125**, 4531-4540.

Jarman, A. P. and Ahmed, I. (1998). The specificity of proneural genes in determining *Drosophila* sense organ identity. *Mech. Dev.* **76**, 117-125.

Jennings, B., Preiss, A., Delidakis, C. and Bray, S. (1994). The *Notch* signalling pathway is required for *Enhancer of split* bHLH protein expression during neurogenesis in the *Drosophila* embryo. *Development* **120,** 3537-3548.

Jonsson, F. and Knust, E. (1996). Distinct functions of the *Drosophila* genes *Serrate* and *Delta* revealed by ectopic expression during wing development. *Dev. Genes Evol.* **206**, 91-101.

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

Kim, J., Irvine, K. and Carroll, S. (1995). Cell recognition, signal induction and symmetrical gene activation at the dorsal-ventral boundary of the developing *Drosophila* wing. *Cell* **82**, 795-802.

Klein, T., Brennan, K. and Martinez Arias, A. (1997). An intrinsic dominant negative activity of *Serrate* that is modulated during wing development in *Drosophila*. *Dev. Biol.* **189**, 123-134.

Klein, T. and Campos-Ortega, J. (1997). *klumpfuss*, a *Drosophila* gene encoding a member of the EGR family of transcription factors, is involved in bristle and leg development. *Development* **124**, 3123-3134.

Klein, T. and Martinez Arias, A. (1998). Interactions among Delta, Serrate and Fringe modulate Notch activity during *Drosophila* wing development. *Development* **125**, 2951-2962.

Kooh, P., Fehon, R. and Muskavitch, M. (1993). Implications of dynamic patterns of *Delta* and *Notch* expression for cellular interactions during *Drosophila* development. *Development* **117**, 493-507.

Kramatschek, B. and Campos-Ortega, J. A. (1994). Neuroectodermal transcription of the *Drosophila* neurogenic genes *E(spl)* and *HLH-m5* is regulated by proneural genes. *Development* **120,** 815-826.

Micchelli, C. A., Rulifson, E. J. and Blair, S. S. (1997). The function and regulation of *cut* expression on the wing margin of *Drosophila*: *Notch*, Wingless and a dominant negative role for *Delta* and *Serrate*. *Development* **124,** 1485-1495.

Muskavitch, M. (1994). *Delta-Notch* signalling and *Drosophila* cell fate choice. *Dev. Biol.* **166**, 415-430.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). *Fringe* modulates *Notch*-ligand interactions. *Nature* **387**, 908-912.

Papayannopoulos, V., Tomlinson, A., Panin, V. M., Rauskolb, C. and Irvine, K. D. (1998). Dorsal-ventral signalling in the *Drosophila* eye. *Science* **281**, 2031-2034.

Parody, T. R. and Muskavitch, M. A. (1993). The pleiotropic function of *Delta* during postembryonic development of *Drosophila melanogaster*. *Genetics* **135,** 527-539.

Robey, E. (1997). Notch in Vertebrates. *Curr. Opin. Genet. Dev.* **7**, 551-557.

Shellenbarger, D. L. and Mohler, J. D. (1975). Temperature-sensitive mutations of the *Notch* locus in *Drosophila melanogaster*. *Genetics* **81,** 143-162.

Speicher, S., Thomas, U., Hinz, U. and Knust, E. (1994). The *Serrate* locus of *Drosophila* and its role in morphogenesis of the wing imaginal discs: control of cell proliferation. *Development* **120**, 535-544.

Villano, J. and Katz, F. (1995). *four-jointed* is required for intermediate growth in the proximal-distal axis in *Drosophila*. *Development* **121**, 2767-2777.

Waddington, C. (1943). The development of some leg genes in *Drosophila*. *J. Genet.* **45**, 29-43.

Weinmaster, G. (1998). *Notch* signalling: direct or what? *Curr. Opin. Genet. Dev.* **8**, 436-442.

Zeng, C., Younger-Shepherd, S., Jan, L. and Jan, Y. (1998). *Delta* and *Serrate* are redundant *Notch* ligands required for asymmetric cell divisions within the *Drosophila* sensory organ lineage. *Genes Dev.* **12**, 1086-1091.