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The establishment of segmentation in the *Drosophila* leg

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SUMMARY

Segmentation is a developmental mechanism that subdivides a tissue into repeating functional units, which can then be further elaborated upon during development. In contrast to embryonic segmentation, *Drosophila* leg segmentation occurs in a tissue that is rapidly growing in size and thus segmentation must be coordinated with tissue growth. I demonstrate that segmentation of the *Drosophila* leg, as assayed by expression of the key regulators of segmentation, the Notch ligands and *fringe*, occurs progressively and I define the sequence in which the initial segmental subdivisions arise. I further demonstrate that the proximal-distal patterning genes *homothorax* and *dachshund* are positively required, while *Distal-less* is unexpectedly negatively required, to establish the segmental pattern of Notch ligand and *fringe* expression. Two *Serrate* enhancers that respond to regulation by *dachshund* are also identified. Together, these studies provide evidence that distinct combinations of the proximal-distal patterning genes independently regulate each segmental ring of Notch ligand and *fringe* expression and that this regulation occurs through distinct enhancers. These studies thus provide a molecular framework for understanding how segmentation during tissue growth is accomplished.

Key words: Leg development, Segmentation, *Drosophila*, *Serrate*, *Delta*, *fringe*, *homothorax*, *dachshund*, *Distal-less*

INTRODUCTION

Segmentation is a developmental mechanism common to a large number of animal species, including insects and vertebrates (Kornberg and Tabata, 1993; McGrew and Pourquie, 1998; Pick, 1998). Segmentation subdivides a tissue into a series of repeating units, whereupon each basic unit can then be further elaborated upon during development. The best-studied example of segmentation is that of the *Drosophila* embryo, where the molecular mechanisms are now well understood (Kornberg and Tabata, 1993; Pick, 1998; Small and Levine, 1991). However, segmentation of the *Drosophila* embryo differs substantially from other organisms, in that segmentation subdivides the entire embryo at once, without accompanying changes in tissue size. By contrast, segmentation of the insect leg, and of the body axes of short germband insects and vertebrates, is fundamentally different in that segmentation occurs in a tissue that is rapidly growing in size (Bishop et al., 1999; de Celis et al., 1998; Gossler and Hrabe de Angelis, 1998; McGrew and Pourquie, 1998; Pick, 1998; Rauskolb and Irvine, 1999). Knowledge of the molecular mechanisms involved in the segmentation of these tissues is more limited.

Most insects have visibly segmented legs. *Drosophila* legs are composed of nine segments; from proximal (closest to the body wall) to distal (tip of the leg) they are the coxa, trochanter, femur, tibia and tarsal segments 1-5 (Fristrom and Chihara, 1978). Each segment is separated from the next by a flexible joint. In recent years, early steps in *Drosophila* leg development have been elucidated, and key genes involved in leg segmentation have been identified (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). Yet, it has remained unclear how a repeating segmental pattern is generated during leg development.

The adult *Drosophila* leg develops during larval stages from a cluster of undifferentiated cells, the leg imaginal disc. The leg disc is divided into anterior and posterior compartments. Signaling from the posterior to anterior compartment induces the expression of Decapentaplegic (DPP) in dorsal anterior cells and Wingless (WG) in ventral anterior cells, with their expression intersecting at the center of the disc (Basler and Struhl, 1994). DPP and WG encode secreted signaling molecules that specify dorsal and ventral cell fates, respectively (Baker, 1988; Brook and Cohen, 1996; Diaz-Benjumea et al., 1994; Held et al., 1994; Struhl and Basler, 1993).

The leg imaginal disc also has a proximal-distal axis. The center of the disc will give rise to the future distal tip of the leg, while progressively more peripheral regions of the disc will give rise to more proximal leg structures (Schubiger, 1971). Importantly, WG and DPP also direct proximal-distal patterning, and act together to regulate the expression of transcription factors expressed in broad domains along the proximal-distal axis of the leg disc, including *homothorax* (*hth*), *dachshund* (*dac*) and *Distal-less* (*Dll*) (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997; Milan and Cohen, 2000; Wu and Cohen, 1999). Leg development is dynamic and the expression profiles of these

genes change as development proceeds (Abu-Shaar and Mann, 1998; Campbell and Tomlinson, 1998; Diaz-Benjumea et al., 1994; Gorfinkiel et al., 1997; Lecuit and Cohen, 1997; Wu and Cohen, 1999). At second instar, the leg disc is divided into two domains: a proximal domain defined by HTH expression and a distal domain defined by DLL expression. At early third instar (~72 hours after egg-laying, AEL) a population of cells expressing DAC arise that lie at an intermediate position between the DLL- and HTH-expressing cells. At mid third instar (~96 hours AEL) clear overlap between the DLL and DAC expression domains is observed. Finally, by late third instar (~120 hours AEL), there is a thin band of cells expressing all three genes, corresponding to the future trochanter.

Importantly, the expression of *hth*, *dac* and *Dll* roughly corresponds to the regions of the leg affected by their absence. Absence of *hth* function results in deletion of proximal leg segments, absence of *dac* function results in deletion of intermediate leg segments, and reduced *Dll* function results in deletion of distal leg segments (Wu and Cohen, 1999; Campbell and Tomlinson, 1998; Cohen and Jurgens, 1989; Gorfinkiel et al., 1997; Mardon et al., 1994). Given that mutations in these broadly expressed genes result in deletions (gaps) of leg segments, and by analogy with embryonic segmentation, they will be referred to, hereafter, as the leg gap genes.

While the function of each leg gap gene spans across several leg segments, localized Notch signaling is required within each leg segment to promote the formation of the boundaries that separate each leg segment and to induce leg growth. Notch is a transmembrane receptor protein (Artavanis-Tsakonas et al., 1999). There are two ligands for Notch in *Drosophila*, Serrate (SER) and Delta (DL) (Artavanis-Tsakonas et al., 1999). In addition, Fringe, a glycosyltransferase, functions to modulate Notch signaling by inhibiting the ability of a cell to respond to SER and potentiating the ability of a cell to respond to DL (Bruckner et al., 2000; Fleming et al., 1997; Moloney et al., 2000; Panin et al., 1997).

SER, DL, and *fringe* are expressed in a segmentally repeated pattern during leg development (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). At late stages of leg development they are expressed in a series of concentric rings within each of the future leg segments, proximal to cells destined to form the segment boundary (i.e. the actual joint). Through mutant clone analysis and ectopic expression studies it has been shown that Notch signaling has a key role in leg segmentation. Clones of cells mutant for *Notch*, Notch ligands or *fringe* result in fusions between leg segments and reduced leg growth (de Celis et al., 1998; Rauskolb and Irvine, 1999). Notch signaling is also sufficient to promote segmentation and growth as ectopic segment borders (joints) and local cell growth are induced when Notch is activated at ectopic sites within the leg (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999).

Importantly, Notch activation must be restricted to a narrow region within each segment for proper leg development. If a constitutively activated form of Notch is expressed continuously across the length of several segments, legs lack segmentation and are shorter (de Celis et al., 1998; Rauskolb and Irvine, 1999). The requirement for a segmentally repeated pattern of Notch activation highlights the importance of establishing appropriately patterned expression of the regulators of Notch activation.

I have identified mechanisms that are responsible for establishing the segmentally repeated expression of the Notch ligands and *fringe*. My results demonstrate that leg segmentation occurs progressively as the leg disc grows, and I establish the temporal and spatial pattern of early steps in segmentation. I further demonstrate that the leg gap genes are key regulators of the segmental pattern of Notch ligand and *fringe* expression, and identify two *Serrate* enhancers that respond to regulation by a leg gap gene. These studies begin to elucidate the molecular mechanisms involved in establishing the repeating segmentation that occurs during *Drosophila* leg development.

MATERIALS AND METHODS

Drosophila strains and generation of clones

Mutant *dac* clones were induced in larvae (48-72 hours AEL at 25°C) of the genotype: *y w hsFLP/+; dac^3 FRT40A/ 2πMyc FRT40A* or, for clones in adult legs, *y w hsFLP/+; dac^3 FRT40A/ y$^+$ FRT40A* (Mardon et al., 1994). Homozygous *dac* mutant leg discs were examined in *dac^3 FRT40A/dac^1 FRT40A* larvae. Mutant *Dll* clones were induced in larvae (60-84 hours AEL at 25°C) of the genotype *y w hsFLP/+; FRT42 Dll^{SA1}/FRT42 armlacZ M* (Abu-Shaar and Mann, 1998).

For ectopic expression studies, ptcGAL4 UASGFP flies were crossed to UAS*dac7c4* (Shen and Mardon, 1997), UAS*Dll*, or UAS*hth12* (Pai et al., 1998), reared at 18°C, and then early to mid third instar larvae were dissected. *SerlacZ1.9* and *SerlacZ2.2* (originally called pCAB70-V-1.9 and pCAB70-I-2.2, respectively) (Bachmann and Knust, 1998) were used to make the stocks *SerlacZ1.9* UAS*dac21M5*/TM6b and *SerlacZ2.2*; UAS*dac21M5*/L14 for the FLP-out experiments. FLP-out clones were generated as described previously (Rauskolb et al., 1999; Rauskolb and Irvine, 1999), using AyGAL4 UASGFP, with clones induced at 48-72 hours AEL at 25°C.

Histology

The following antibodies were used: mouse anti-DAC (DSHB), mouse anti-DLL (Duncan et al., 1998), rat anti-DLL (Wu and Cohen, 2000), rabbit anti-HTH (Kurant et al., 1998), rabbit anti-SER (Thomas et al., 1991), rat anti-SER (Papayannopoulos et al., 1998), mouse anti-DL (DSHB), goat anti-β-gal (Biogenesis) and rabbit anti-MYC (Santa Cruz Biotechnology). Antibody staining was performed as described (Panin et al., 1997; Rauskolb and Irvine, 1999). *fringe* expression was detected by in situ hybridization to mRNA (Rauskolb and Irvine, 1999).

RESULTS

SER expression during leg development is progressive

In theory, nine segmental units could be established at one time once leg growth is complete, similar to segmentation of the *Drosophila* embryo. However, several observations suggest that leg segmentation may be progressive. First, temperature-shifts of a conditional *Notch* allele at different stages of development interfere with the formation of distinct joints (Shellenbarger and Mohler, 1978). Second, the rings of expression of a reporter gene construct that responds to Notch activation are established sequentially during leg development (de Celis et al., 1998). Although these studies indicate a

temporal response to Notch activation, they do not differentiate as to whether this occurs because the expression of the regulators of Notch activation, SER, DL and *fringe*, is established progressively or because of effects downstream of Notch activation.

To determine whether leg segmentation is initiated progressively upstream of Notch activation, I examined the expression of SER, DL and *fringe*, the earliest known indicators of a segmental pattern and the key regulators of Notch activation and leg segmentation. The initial focus has been on SER because a good antibody is available and because *SerlacZ* reporter gene constructs that represent a fraction of the SER expression profile exist. SER expression at different developmental time points was compared with that of the leg gap genes, because their expression has been well described and their relationship to presumptive leg segments established. In most cases, this made it possible to ascribe the rings of SER expression to particular leg segments. Moreover, these comparative expression studies suggested regulatory relationships that were then tested experimentally.

At ~72 hours AEL (early third instar) there is a single proximal ring of SER expression. This ring of expression arises within the presumptive coxa, as it lies just proximal to DAC-expressing cells, and within cells expressing HTH (Fig. 1A; Fig. 2A,B). A few hours later, at ~78 hours AEL, a second ring of SER expression arises just distal to the first (Fig. 1B). This expression arises within the femur, within cells expressing DAC; however, not all DAC-expressing cells express SER – only proximal ones near the HTH expression domain (Fig. 2C). By ~84 hours AEL, two additional sites of SER expression are detectable, both within DLL-expressing cells of the presumptive tarsus (Fig. 1C). The more proximal of these is a

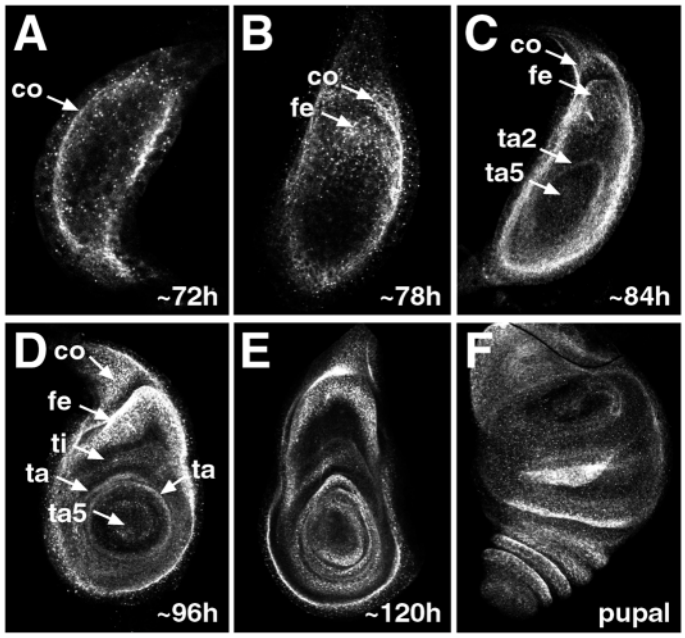

Fig. 1. Expression of SER is established progressively. (A) In early third instar leg discs (~72 hours AEL), a ring of SER expression is detected in the coxa. (B) At ~78 hours AEL, a new ring of SER expression arises in the femur. (C) By ~84 hours AEL, SER expression is detected in four domains, two of which are in the tarsal region (ta2 and ta5). (D) By mid third instar (~96 hours AEL), SER is expressed in at least six prospective segments. It is unclear whether the new tarsal ring corresponds to ta1 or ta3; therefore, both the ta2 and the new ring are designated as ta. (E,F) Ultimately, by late third instar (~120 hours AEL) SER is expressed in one ring per segment and expression continues during pupal stages. co, coxa; fe, femur; ta2,tarsal segment 2; ta5, tarsal segment 5; ti, tibia.

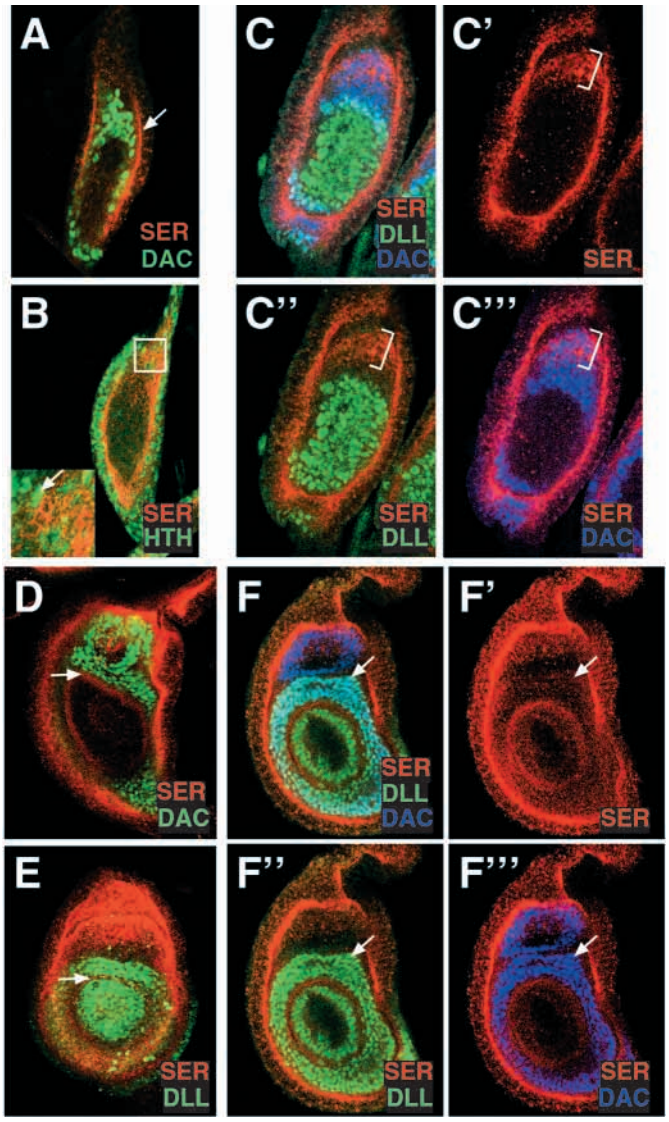

Fig. 2. Expression of SER relative to the leg gap genes. SER expression (red) was compared with that of HTH, DAC, and DLL. (A,B) The coxa ring of SER (arrow) arises in cells proximal to those expressing DAC (A, green), within cells expressing HTH (B, green). SER expression is at the cell membrane and HTH expression is nuclear, so overlap in expression is not seen in all focal planes. Arrow in inset points to two cells obviously expressing both HTH and SER. (C-C''') Femur SER expression (bracket) arises within proximal DAC-expressing cells (blue). DLL expression (green) does not overlap with the femur SER expression. (D,E) At early-mid third instar, two sites of SER expression arise in the tarsus. A ring of expression (arrow) is observed distal to cells expressing DAC (D, green), but within cells expressing DLL (E, green). (F-F''') Expression of SER in the tibia (arrow) arises in cells expressing both DAC (blue) and DLL (green). DAC and DLL overlap in turquoise in C and F.

ring of expression within the DLL domain, immediately distal to cells expressing DAC (Fig. 2D,E). I have tentatively assigned this ring to tarsal segment 2, based on the reported expression domains of DAC and DLL later in development (Milan and Cohen, 2000). SER expression is also observed in a central spot in the most distal region of the leg disc, in tarsal segment 5 (Fig. 1C; Fig. 2D,E). By ~96 hours AEL (mid third instar) six sites of SER expression are detectable; a tibia ring has been added as well as an additional tarsal ring (Fig. 1D). The tibia ring of expression arises in cells expressing both DAC and DLL (Fig. 2F). Late in larval development (~120 hours AEL; late third instar) there appears to be a ring of SER expression in each of the prospective leg segments (Fig. 1E) (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999), with expression continuing to pupal stages (Fig. 1F) (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999).

Early SER expression is regulated by the leg gap genes

Mutation of *hth*, *dac* or *Dll* results in deletion of leg segments (Campbell and Tomlinson, 1998; Cohen and Jurgens, 1989; Mardon et al., 1994; Wu and Cohen, 1999). Moreover, clones of cells mutant for any of these genes can be associated with leg segment fusions (Fig. 3D,G) (Campbell and Tomlinson, 1998; Wu and Cohen, 1999). Although the mechanism by which mutation of a leg gap gene results in leg segment fusions and tissue loss is not known, one possibility is that it reflects the requirement for these genes in establishing the striped expression of the Notch ligands and *fringe*. To test this hypothesis, I examined the influence of ectopic or absence of expression of DAC, HTH and DLL on SER expression during leg development.

DAC positively regulates SER expression

The femur ring of SER expression arises within cells expressing DAC. *dac* mutant flies survive to adulthood (Mardon et al., 1994), and thus SER expression could be examined in leg discs from larvae entirely mutant for *dac*. After 78 hours of development, when the femur ring of SER expression is normally present, SER expression in the coxa ring is unaffected, but expression in the femur ring is greatly reduced (Fig. 3F). To rule out the possibility that the femur ring is simply absent because these cells are missing in *dac* mutants (Mardon et al., 1994), I also analyzed *dac* mutant clones for their effects on femur SER expression. *dac* mutant clones are of comparable size with wild-type twin clones, indicating that *dac* mutant cells do not have a growth disadvantage (data not shown). Importantly, SER expression is cell-autonomously eliminated from *dac* mutant cells located in the femur (Fig. 3C). Moreover, *dac* is required autonomously for expression of SER in the tibia, although some nonautonomous rescue by neighboring wild-type cells is also observed (Fig. 3E). The absence of SER expression in *dac* mutant clones in the femur and tibia is consistent with the segment fusions observed in *dac* mutant clones in adult legs (Fig. 3D and data not shown). Together, these results demonstrate that DAC is an important positive regulator of SER expression in the femur and tibia. However, as SER is not expressed by all DAC-expressing cells, other factors must also influence SER expression within the DAC domain.

To determine whether DAC is also sufficient to promote SER expression, DAC was ectopically expressed along the anteroposterior (AP) axis of the developing leg (ptcGAL4 UAS*dac*). At ~78 hours AEL, when SER is normally expressed in two proximal rings, ectopic expression of DAC cell-autonomously induces SER expression along the entire AP axis of the leg disc (compare Fig. 3A with Fig. 1B), resulting in leg

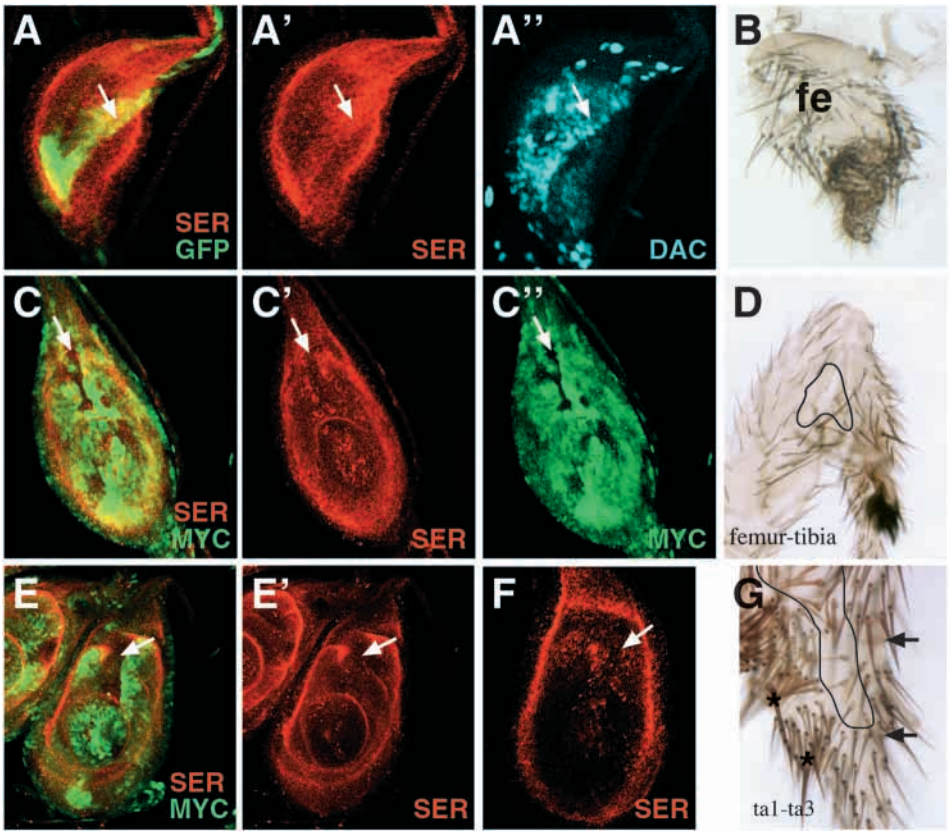

Fig. 3. DAC positively regulates SER expression. (A-A'') ptcGAL4 UAS*dac* UASGFP results in a cell autonomous induction of SER expression (red, arrow) in early third instar leg discs. (B) ptcGAL4 UAS*dac* adult leg with segmental fusions. fe, femur. (C-C'') *dac* mutant clone showing a cell autonomous loss of SER expression in the femur (arrow). (E-E') *dac* mutant clone in the tibia results in a cell autonomous loss of SER expression, except at the proximal edge of the clone (arrow). (F) *dac* homozygous mutant leg disc with greatly reduced SER expression in the femur (arrow). (D,G) *dac* mutant clones (outlined) cause segment fusions in adult legs. (D) Femur-tibia fusion. (G) Fusion extending from tarsal segments1-3. Arrows indicate where normal joints would lie (partially visible by upper arrow); asterisks denote apical bristles, which normally lie just proximal to tarsal segment borders.

segment fusions in the adult leg (Fig. 3B). Thus, DAC positively regulates SER expression during early leg development. It was initially surprising to observe ectopic SER expression induced in the domain of the leg where DAC is already expressed; however, ptcGAL4 drives the expression of DAC well above endogenous levels (Fig. 3A). Importantly, this indicates that the effect of DAC on SER expression is not mediated simply by repression of HTH or DLL by DAC, as these genes are not expressed significantly in the DAC domain at this stage. Together, these studies demonstrate that DAC is necessary and sufficient to establish SER expression in intermediate segments during leg development.

HTH autonomously represses and nonautonomously induces early SER expression

HTH is required for the formation of the most proximal leg segments and is expressed in the coxa and trochanter (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). The first ring of SER expression arises in the coxa and is the first sign of segmentation of the leg disc (Fig. 1A, Fig. 2A,B). To examine the influence of HTH on SER expression, HTH was ectopically expressed along the AP axis of the developing leg (ptcGAL4 UAS*hth*).

Overexpression of HTH in proximal cells has no effect on the coxa ring of SER expression (Fig. 4A), consistent with the observation that the coxa ring of SER arises in cells that already express HTH. By contrast, ectopic HTH represses the expression of the femur ring of SER, and induces SER expression in neighboring cells in more distal regions of the leg (Fig. 4B). The phenotypic consequence of this is that the leg field is now split in two, with two rings of SER forming side by side and resulting in bifurcated adult legs (Fig. 4B,C). It has been reported that HTH can inhibit DAC and DLL expression cell autonomously, and HTH can induce DAC expression nonautonomously (Abu-Shaar and Mann, 1998; Goto and Hayashi, 1999; Wu and Cohen, 1999). Thus, the regulation of femur SER expression by HTH may be an indirect consequence of its effects on DAC expression. Indeed, in those cells in which femur SER expression is autonomously repressed, DAC expression is also repressed (Fig. 4D). As *dac* is positively required for SER expression in the femur (Fig. 3C), this can account for the autonomous repression of SER by HTH. The nonautonomous induction of SER expression by HTH appears more complicated. On the anterior side of the ptcGAL4 stripe, DAC expression is induced, which could thus account for the induction of SER (Fig. 4D). By contrast, on the posterior side of the ptcGAL4 stripe, DAC expression is not detectably induced in the cells ectopically expressing SER (Fig. 4D). Thus, in this instance, SER expression appears to be induced nonautonomously by HTH in a DAC-independent manner.

DLL represses tarsal SER expression

The distal rings of SER expression arise within cells expressing DLL. To determine whether DLL regulates SER expression, I examined the expression of SER in *Dll* mutant clones in early leg discs. *Dll* mutant clones will sort out to proximal regions of the disc as *Dll* also affects cell adhesive properties, but occasionally mutant cell will become 'trapped' in distal regions of the leg (Wu and Cohen, 1999). Strikingly, I observed that SER expression was derepressed cell autonomously in *Dll* mutant clones located anywhere within the DLL domain (Fig. 5D; data not shown). Thus, DLL must normally act to repress SER expression, an unexpected result given that SER is normally expressed in the tarsus.

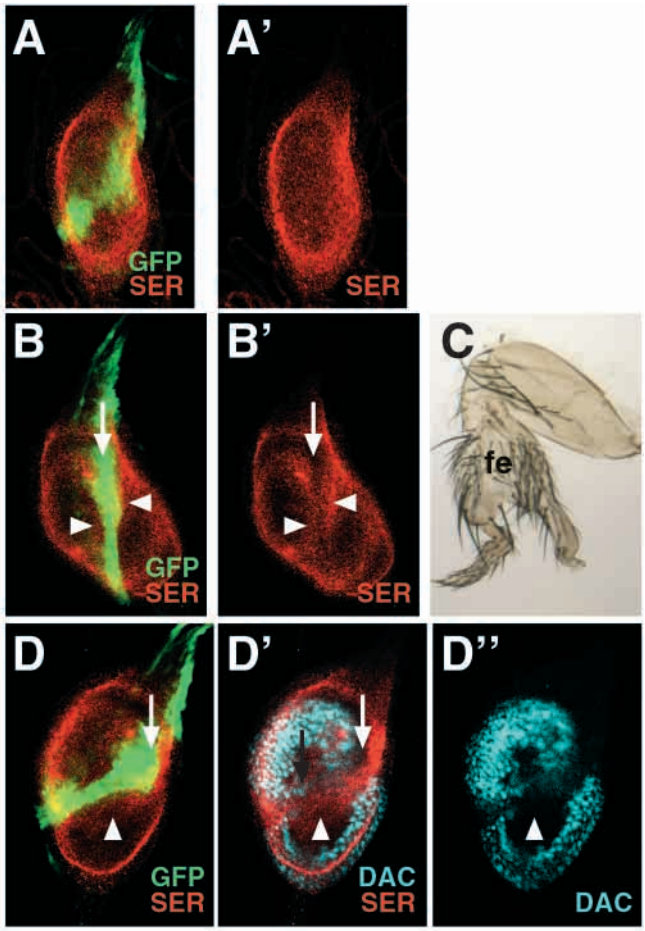

Fig. 4. HTH regulates early SER expression both cell autonomously and nonautonomously. All panels are leg discs from ptcGAL4 UAS*hth* UASGFP larvae; SER in red. (A,A') Ectopic expression of HTH does not affect SER expression in the coxa. (B,B') Ectopic expression of HTH cell autonomously represses SER expression in the femur (arrow), and cell nonautonomously induces SER (arrowheads). (C) Adult leg displaying bifurcation at the level of the femur (fe). (D-D'') Ectopic expression of HTH cell autonomously represses DAC expression (white arrow). In the anterior compartment, ectopic HTH results in an induction of DAC expression (black arrow), while in the posterior compartment, ectopic HTH does not affect DAC expression in those cells in which SER was induced (arrowhead).

To further examine the repression of SER expression by DLL, I assayed the consequences of ectopic DLL expression. Expression of DLL along the AP axis of the developing leg (ptcGAL4 UAS*Dll*) disrupts the femur ring of SER (Fig. 5A). The repression of SER expression in the femur is likely to be indirect, however, as a concomitant repression of DAC expression occurs (Fig. 5A). Similarly, DLL can repress SER expression in the coxa, but this effect may also be indirect through effects of DLL on HTH expression (Fig 5B; data not shown) (Abu-Shaar and Mann, 1998). Importantly though, SER expression is also repressed by DLL in tarsal cells, outside the normal HTH and DAC expression domains (Fig. 5B). The

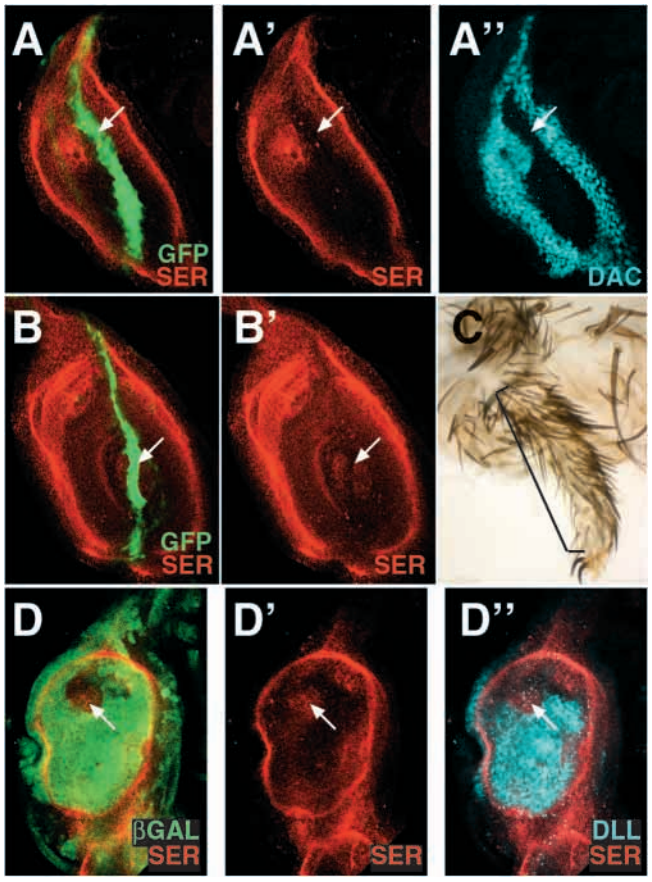

Fig. 5. DLL represses SER expression. (A-A'') ptcGAL4 UAS*Dll* UASGFP early third instar leg discs. Ectopic DLL expression in the femur (arrow) results in a cell autonomous loss of SER and DAC expression. (B,B') ptcGAL4 UAS*Dll* UASGFP results in repression of SER expression in tarsal segment 5 (arrow). (C) ptcGAL4 UAS*Dll* adult leg with tarsal segment fusions (bracket). (D-D'') *Dll Minute* clone results in the cell autonomous induction of SER expression within the tarsus (arrow).

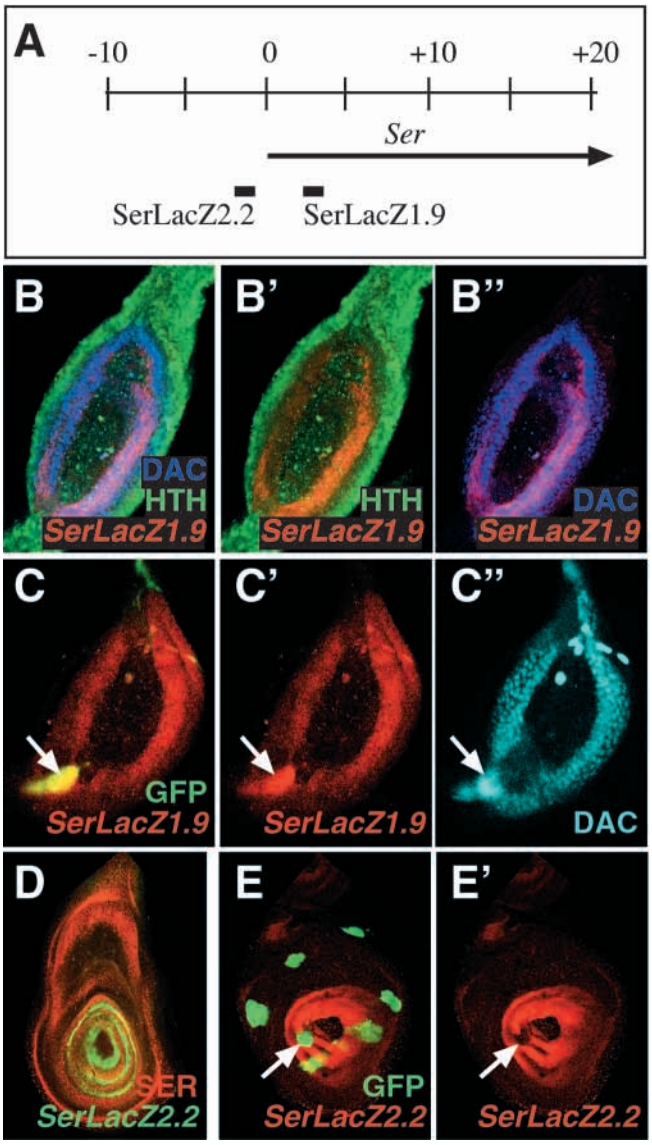

Fig. 6. DAC acts through minimal *Ser* enhancer elements. (A) Schematic of the *Ser* locus with genomic regions used for the *lacZ* reporter constructs indicated as *SerlacZ2.2* (2.2 kb fragment) and *SerlacZ1.9* (1.9 kb fragment) (Bachmann and Knust, 1998). (B-B'') Expression of *SerlacZ1.9* (red) is first detected in the prospective femur. β-Galactosidase expression overlaps with DAC (blue) and lies distal to HTH (green). (C-C'') FLP-out clones of *dac* (marked with GFP, green) result in cell autonomous induction of *SerlacZ1.9* expression (red, arrow). (D) Expression of *SerlacZ2.2* in tarsal segments 1-4 (green) is complementary to endogenous SER expression (red). (E,E') FLP-out clones of *dac* (marked with GFP, green) result in cell autonomous repression of *SerlacZ2.2* expression (red, arrow).

expression of DLL along the AP axis of the leg also results in segment fusions throughout the tarsus (Fig. 5C). Thus, DLL is both necessary and sufficient to repress SER expression, and it appears that high levels of DLL expression can override positive regulators of SER expression within the tarsus.

DAC acts through sequences present in minimal SER reporter gene constructs

The results presented above establish that all of the known leg gap genes, HTH, DAC and DLL, are key regulators of SER expression. To begin to identify the actual sequences through which the upstream regulators act, I have begun to investigate the regulation of SER reporter gene constructs, identified previously by Bachmann and Knust (Bachmann and Knust, 1998), and reported to give SER-like expression in proximal versus distal domains of the leg disc. The sequences in these reporter constructs are from non-overlapping regions of the *Ser* locus (Fig. 6A) (Bachmann and Knust, 1998).

SerlacZ1.9

SerlacZ1.9 contains 1.9 kb from the *Ser* locus fused to *lacZ* (Bachmann and Knust, 1998). *SerlacZ1.9* expression is first detected at early third instar in a single ring (Fig. 6B), which corresponds to the femur ring of endogenous SER, as it lies just distal to the HTH domain and within the DAC domain (Fig. 6B). Thus, this reporter gene construct appears to contain the sequences required for SER expression in the femur, but lacks the sequences necessary for expression in the coxa. Moreover, because of the overlap with DAC expression,

SerlacZ1.9 may contain the sequences required for *Ser* regulation by DAC. By the end of the third instar, expression of *SerlacZ1.9* continues in the femur but is also detected in a segmentally repeated pattern in most leg segments (data not shown).

To test whether early *SerlacZ1.9* expression is regulated by DAC, I examined the effects of ectopic DAC expression. Clones of cells ectopically expressing DAC express *SerlacZ1.9* at high levels in all regions of the disc, including where endogenous DAC is expressed (Fig. 6C; data not shown). These results argue that *SerlacZ1.9* is a *Ser* enhancer containing regulatory elements that are responsive to positive regulation by DAC.

SerlacZ2.2

SerlacZ2.2 contains 2.2kb from the *Ser* locus fused to *lacZ* (Bachmann and Knust, 1998). Expression is restricted to the distal region of the developing leg in a ring in each of tarsal segments 1-4 (Fig. 6D) (Bachmann and Knust, 1998; Buckles et al., 2001). *SerlacZ2.2* expression begins at mid third instar, later than *SerlacZ1.9* expression is initially detected (data not shown). The expression of *SerlacZ2.2* and DAC are complementary; *SerlacZ2.2* is expressed entirely within the DLL domain (data not shown).

Surprisingly, a comparison of *SerlacZ2.2* and SER expression at both mid third instar and pupal stages has revealed that expression of *SerlacZ2.2* is complementary to the endogenous SER pattern in the distal leg (Fig. 6D) (Buckles et al., 2001). Thus, although *SerlacZ2.2* contains the sequences necessary for restricting expression to the distal leg, sequences required for directing expression to the appropriate domain of each leg segment appear absent.

To investigate whether DAC acts to restrict *SerlacZ2.2* expression to the distal regions of the leg, I examined FLP-out clones of cells ectopically expressing DAC. Indeed, DAC represses *SerlacZ2.2* expression in the distal leg (Fig. 6E) and has no effect on *SerlacZ2.2* expression elsewhere. This observation contrasts with the results obtained with SER expression in the femur and *SerlacZ1.9*, which are both positively regulated by DAC.

DL and *fringe* expression occur progressively during leg development and are regulated by DAC

Like SER, DL and *fringe* are expressed in a series of segmentally repeated rings during leg development. Late in leg development, DL expression overlaps that of SER, but also extends, in some segments, somewhat into regions of the disc where SER is not expressed (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). *fringe* expression appears coincident with SER expression in all leg segments (Rauskolb and Irvine, 1999). However, the expression of DL and *fringe* has not been characterized at early stages of leg development.

To determine whether DL and *fringe* expression are, like SER, progressive during leg development and how their profile compares with that of SER, their expression was examined at various stages. Indeed, both DL and *fringe* expression arise progressively, in a similar step-wise sequence as that observed for SER (Fig. 7A-C,G,H and data not shown). Interestingly, SER and DL expression in the coxa are coincident in early third instar leg discs (Fig. 7D). Thus, even at the earliest stages of leg development, the cells express both Notch ligands. This suggests that these genes overlap in expression throughout all of leg development, as they have been observed to do in most segments at late stages (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). The only notable difference among DL, SER and *fringe* is that SER and *fringe* expression arises in the most distal region of tarsal segment 5, which will ultimately give rise to the pretarsus, while DL expression encircles this domain (Fig. 7B and data not shown) (de Celis et al., 1998; Rauskolb and Irvine, 1999). The nearly identical expression patterns suggest that DL and *fringe* may also be regulated by the leg gap genes. Indeed, when DAC was ectopically expressed along the AP axis of the developing leg (ptcGAL4 UAS*dac*), both DL and *fringe* expression were induced in an apparently cell autonomous fashion (Fig. 7E,F,I), paralleling the positive regulation of SER by DAC (Fig. 3).

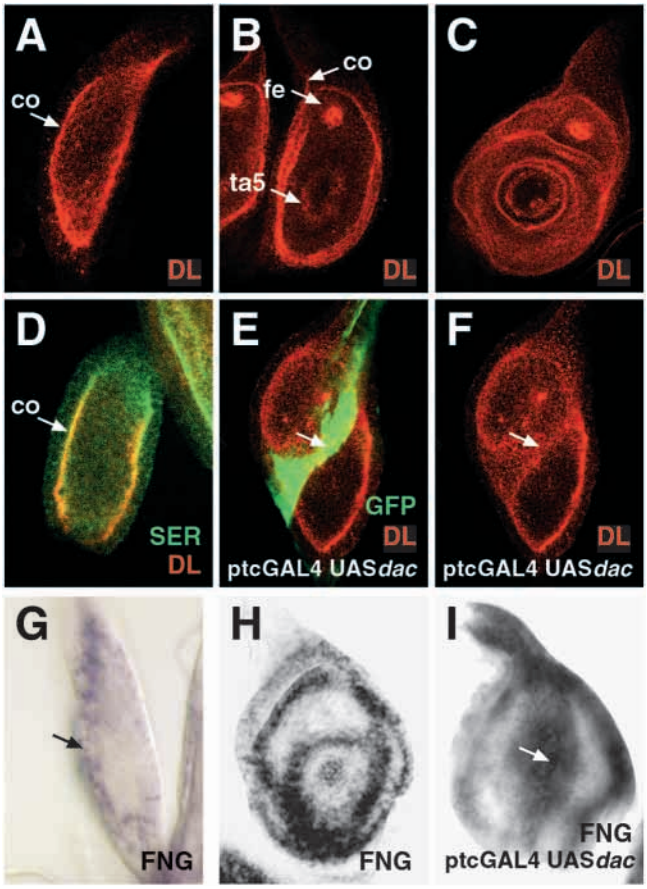

Fig. 7. DAC regulates the progressive expression of DL and *fringe*. (A-C) DL expression (red) occurs progressively with more rings of expression added as development proceeds. (D) DL (red) and SER (green) expression are coincident within the coxa of early third instar leg discs (arrow). (E,F) ptcGAL4 UAS*dac* UASGFP early third instar leg disc. DL expression is cell autonomously induced by DAC (arrow). (G) *fringe* expression at early third instar. Expression is visible in a single proximal ring (arrow). (H) *fringe* expression at early-mid third instar. Expression is observed in three rings and a central spot. (I) ptcGAL4 UAS*dac* expression results in the induction of *fringe* expression in a stripe along the AP axis of the developing leg (arrow).

DISCUSSION

Progressive segmentation of the *Drosophila* leg

In recent years the key role of the Notch signaling pathway in the segmentation and growth of the *Drosophila* leg has been established. Notch signaling must be localized within each leg segment to promote the formation of boundaries (joints) that separate each leg segment and to induce leg growth. This requirement for a segmentally repeated pattern of Notch activation is accomplished by restricting the expression of the regulators of Notch activation, SER, DL and *fringe*, to one ring per segment. By examining the expression of the Notch ligands and *fringe* during leg development, it has been possible to determine the progressive order in which leg segmentation is established (summarized in Fig. 8). At early third instar, a single ring of SER, DL and *fringe* expression is present within the coxa. The next ring to arise is located within the presumptive femur. At mid third instar, expression arises within presumptive tarsal segments 2 and 5. Subsequent expression is observed in the tibia and more tarsal segments, such that ultimately, by the end of third instar, a ring of expression is present in each presumptive leg segment, adjacent to each prospective leg segment border. Thus, segmentation of the *Drosophila* leg occurs progressively and in a reproducible pattern.

Previous studies investigating the expression of a reporter gene (E(spl)mβ-CD2) regulated downstream of Notch activation led to the conclusion that the first segment boundary to form was between tarsal segments 4 and 5 (de Celis et al., 1998). Additional rings of expression were then observed in the tarsus and then eventually in all leg segments. This led to the suggestion that the first segmental boundaries to form correspond to the most distal segments. However, further examination of this reporter gene indicates that expression is observed in proximal cells prior to expression within the tarsus [i.e. fig. 7 in de Celis et al. (de Celis et al., 1998)]. Moreover, temperature shifts of a conditional *Notch* allele at different stages of development demonstrated that the temperature-sensitive period for Notch in proximal segmentation occurs before that in tarsal segmentation (Shellenbarger and Mohler, 1978). These studies and the data presented here lead to the conclusion that leg segmentation does not occur in a simple distal to proximal order, nor proximal to distal order, nor are the most proximal and distal segments established first and other segments added by intercalation, as has been previously

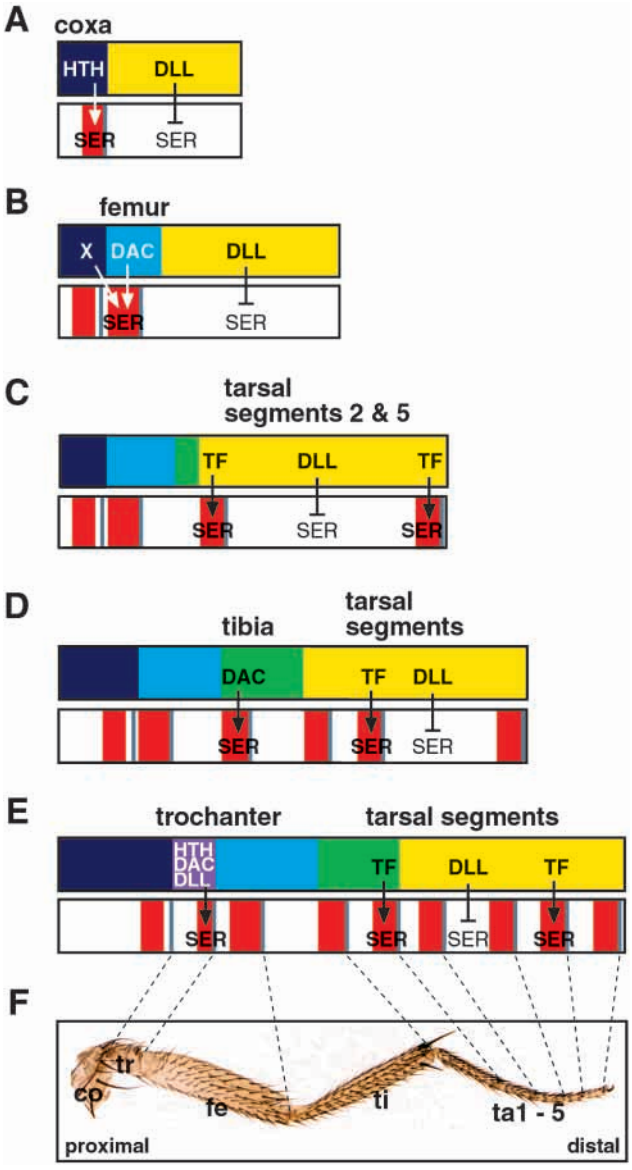

Fig. 8. Summary model for the establishment of segmentation in the *Drosophila* leg. Although SER and leg gap gene expression occurs within the same cells, their expression is depicted here as two separate bars. For each panel, the top bar incicates the expression of the leg gap genes and the bottom bar denotes where SER expression (in red) is induced relative to this expression, with proximal on the left and distal on the right. Expression of HTH is in dark blue; DLL in yellow; DAC in light blue; DAC and DLL overlap in green; and HTH, DAC and DLL overlap in purple. Position of eventual leg segment borders shown in gray. Similar regulatory interactions are thought to induce segmental DL and *fringe* expression. Note that the model, as drawn here, depicts an increase in leg disc size during development, but the amount of growth and expression domains are not necessarily to scale. See text for further details of model. (A) Early third instar (~72 hours AEL). HTH induces SER expression within the coxa, while DLL represses SER expression in more distal regions. (B) Early third instar (~78 hours AEL). DAC induces SER expression within the femur, while HTH-expressing cells produce a signal (X) that nonautonomously induces SER within the femur. (C) Early-mid third instar (~84 hours AEL). DLL represses SER expression, yet two sites of expression are observed within the tarsus. Other genes expressed within the tarsus (tarsal factors, TF), such as SS and/or BAB, may induce expression here. At early third instar SS expression overlaps with DLL, while later DLL expression extends more proximally. BAB has graded expression in tarsal segments 1-4. (D) Mid third instar (~96 hours AEL). DAC induces SER expression within the tibia, perhaps by overcoming repressive effects of DLL. New tarsal rings are also induced, depicted here in tarsal segment three; however, the precise segmental order in which tarsal expression arises is not known. (E) Late third instar (~120 hours AEL). HTH, DAC and DLL are hypothesized to function together to induce SER within the trochanter. All tarsal segments also now express SER. (F) Adult leg, with leg segment borders shown relative to the schematic shown in E. Leg segment borders (the joints) form just distal to the sites of Notch ligand expression, and do not always correspond to boundaries of leg gap gene expression. co, coxa; fe, femur; ta1-5,tarsal segments 1-5; ti, tibia; tr, trochanter. The claw forms at the distal tip of the leg.

suggested (Goto and Hayashi, 1999; Schubiger, 1974). Rather, the establishment of *Drosophila* leg segmentation occurs in a complex sequence (Fig. 8).

Establishment of segmentation by the leg gap genes

A general theme in patterning during development is the subdivision of tissues initially by genes expressed in broad, partially overlapping domains, which through combinatorial control, subsequently regulate the expression of downstream genes to generate a repeating pattern (Irvine and Rauskolb, 2001; Lawrence and Struhl, 1996). The studies presented here demonstrate that leg segmentation follows this same theme. The 'leg gap genes' HTH, DAC, and DLL are expressed in broad domains in the leg disc that encompass more than a single segment (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997; Wu and Cohen, 1999). Initially expression of these genes is largely nonoverlapping, but as the leg disc grows, the expression patterns of the leg gap genes change such that five different domains of gene expression are established (Fig. 8). The analysis of the regulation of Notch ligand and *fringe* expression during leg development reveals two fundamental aspects of leg development. First, these leg gap genes are key components in regulating the expression of the molecules controlling segmentation. Indeed, the effect of these leg gap genes on leg segmentation and growth can be accounted for by their regulation of SER, DL and *fringe* expression. Second, the expression of each ring of SER, DL and *fringe* is controlled by its own unique combination of regulators, apparently acting through independent enhancers.

How do these three transcription factors regulate the formation of nine segments? As the requirements for and the expression of the leg gap genes encompasses all leg segments, I think it unlikely that there are additional leg gap genes yet to be identified. Rather, a collection of distinct combinatorial approaches is used to establish a segmental pattern of SER, DL and *fringe* expression (Fig. 8).

In early third instar leg discs, there are two domains of gene expression, proximal cells express HTH and distal cells express DLL. HTH autonomously promotes the expression of SER, while DLL may prevent expression more distally, giving rise to a ring of expression in the coxa (Fig. 8A). Additionally, DLL-expressing cells may signal to the HTH-expressing cells to restrict SER expression to the distal edge of the HTH domain. As the leg disc grows, cells in an intermediate position, lying between the HTH and DLL domains, begin to express DAC (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997; Wu and Cohen, 1999). DAC, as shown here, is both necessary and sufficient to induce the expression of SER, DL and *fringe* within the femur (Fig. 8B). As they are not expressed in all DAC-expressing cells, other factors appear to be required to promote their expression in the proximal femur. The nonautonomous induction of SER expression by HTH suggests that this may be accomplished by a signal (X) emanating from the neighboring HTH-expressing cells (Fig. 8B). By mid third instar stages, expression of SER, DL and *fringe* is also observed in tarsal segments 2 and 5, within cells expressing DLL but not DAC. Given that DLL is necessary and sufficient to repress their expression, SER, DL and *fringe* expression within the tarsus appears to be induced by a mechanism that overrides the repressive effects of DLL (Fig. 8C). Subsequently, expression of SER, DL and *fringe* is observed within the tibia, in cells expressing both DAC and DLL (Fig. 8D). DAC is necessary for expression of SER within the tibia, and its role here may be to overcome the repressive effects of DLL. It is also worth noting that the tibia ring of expression is not established at the time when cells first express both DAC and DLL (compare Fig. 2C with 2F). This may be because DAC levels may not be sufficiently high enough to overcome the repression by DLL. Clearly levels of DAC expression are critical because simply increasing DAC levels, as in the ptcGAL4 UAS*dac* experiments, is sufficient to promote SER expression in cells already expressing endogenous levels of DAC (Fig. 3A). This observation can be explained if high levels of DAC expression in cells already expressing DAC override the function of inhibitory regulators of SER expression, such as DLL, where the expression of these genes overlap. Although I have not investigated late stages of leg segmentation, it has been noted that HTH, DAC and DLL are co-expressed in the presumptive trochanter late in leg development (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). I thus hypothesize that SER, DL and *fringe* expression is established by the combined activities of the three leg gap genes in the trochanter (Fig. 8E).

Although the studies presented here have focused on the regulation of SER expression, I think that not only SER, but also DL and *fringe*, receive primary regulatory input from the leg gap genes. I have shown that DL and *fringe* expression, like SER, is positively regulated by DAC. Moreover, *Dl* and *fringe* mutants have stronger leg segmentation phenotypes than *Ser* mutants, and thus DL and *fringe* expression cannot simply be regulated downstream of *Ser*. The identification of two separate *Ser* enhancers, directing expression in the proximal versus distal leg, argues against SER being regulated downstream of *Dl* and *fringe*. Thus, the simplest model is that expression of all three genes is regulated directly by the leg gap genes. The regulation of SER, DL and *fringe* expression in each segment appears to occur through independent and separable enhancer elements, supported by the analysis of the *Ser* reporter genes. This is reminiscent of what occurs during *Drosophila* embryonic segmentation, where separable enhancer elements direct different stripes of pair-rule gene expression (Pick, 1998; Small and Levine, 1991).

Importantly, Notch signaling may actually coordinate progressive segmentation of the leg with leg growth. For example, in early leg discs there is a single ring of SER expression within the coxa, in HTH-expressing cells immediately adjacent to DAC-expressing cells (Fig. 2A,B). However, by the time the femur ring arises, the coxa ring of SER expression has been displaced and is no longer within cells immediately adjacent to the DAC-expressing cells; rather, there are HTH-expressing cells lying in between that do not express SER (Fig. 2C). Thus, I postulate that once SER, DL and *fringe* expression is established within the coxa, Notch is activated, which promotes local cell proliferation, thereby displacing the coxa ring. This then allows for the femur ring of expression to be established in cells that are not immediately adjacent to the coxa expression ring. This mechanism also requires that once a ring of ligand expression is established in a particular segment, this expression must be maintained such that it is not influenced by later alterations in relation to leg gap gene expression. This maintenance could be accomplished by a feedback loop between Notch activation and ligand

expression, similar to what has been observed during late wing development, where Notch activation cell autonomously represses ligand expression and nonautonomously induces ligand expression in flanking cells by regulating the expression of a signaling molecule (Irvine and Vogt, 1997). Preliminary studies have indicated that Notch activation can influence Notch ligand expression in the developing leg (C.R., unpublished observations).

Tarsal segmentation

Most of the tarsus of the *Drosophila* leg derives from cells expressing DLL, but not DAC or HTH (Campbell and Tomlinson, 1998; Gorfinkiel et al., 1997; Lecuit and Cohen, 1997). The studies presented here have surprisingly shown that DLL actually represses Notch ligand expression. This negative regulatory role for DLL contrasts with the positive promoting role of DAC and HTH, and further indicates that a distinct molecular mechanism must promote segmentation within the tarsus. One key gene is *spineless-aristapedia* (*ss*), as simple, unsegmented tarsi develop in *ss* mutant flies (Duncan et al., 1998). Moreover, *ss* regulates the expression of *bric-à-brac* (*bab*), which is also required for the subdivision of the tarsus into individual segments (Duncan et al., 1998; Godt et al., 1993). Together, *ss* and *bab* must, in some way, ultimately overcome the repression of Notch ligand and *fringe* expression by DLL. If the sole function of *ss* and *bab* is to overcome the inhibitory effects of DLL, then in the absence of *ss* and/or *bab*, SER expression is expected to remain repressed.

Intriguingly, the only notable variation between insect species is in the number of tarsal segments, with an unsegmented tarsus believed to be the ancestral state (Boudreaux, 1987). Thus, the combinatorial regulation of segmentation by the leg gap genes may represent an ancient mechanism common to all insect species, a hypothesis supported by the conserved expression of HTH, DAC and DLL in the developing legs of many insect species (Abzhanov and Kaufman, 2000; Jockusch et al., 2000; Panganiban et al., 1994).

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REFERENCES

Abu-Shaar, M. and Mann, R. S. (1998). Generation of multiple antagonistic domains along the proximal-distal axis during *Drosophila* development. *Development* **125**, 3821-3830.

Abzhanov, A. and Kaufman, T. C. (2000). Homologs of Drosophila appendage genes in teh patterning of arthropod limbs. *Dev. Biol.* **227**, 673-689.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: Cell fate control and signal integration in development. *Science* **284**, 770-776.

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

Baker, N. E. (1988). Transcription of the segment-polarity gene *wingless* in the imaginal discs of *Drosophila*, and the phenotype of a pupal-lethal *wingless* mutation. *Development* **102**, 489-497.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. *Nature* **368**, 208-214.

Bishop, S. A., Klein, T., Martinez Arias, A. and Couso, J. P. (1999). Composite signalling from Serrate and Delta establishes leg segments in Drosophila through Notch. *Development* **126**, 2993-3003.

Boudreaux, H. B. (1987). Arthropod Phylogeny, with special reference to insects. Malabar, FL: Robert E. Krieger Publishing.

Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila Leg. *Science* **273**, 1373-1377.

Bruckner, K., Perez, L., Clausen, H. and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* **406**, 411-415.

Buckles, G. R., Rauskolb, C., Villano, J. and Katz, F. N. (2001). *Four-jointed* interacts with *dachs*, *abelson*, and *enabled* and feeds back onto the *Notch* pathway to affect growth and segmentation in the *Drosophila* leg. *Development* **128**, 3533-3542.

Campbell, G. and Tomlinson, A. (1998). The roles of the homeobox genes *aristaless* and *Distal-less* in patterning the legs and wings of *Drosophila*. *Development* **125**, 4483-4493.

Cohen, S. M. and Jurgens, G. (1989). Proximal-distal pattern formation in Drosophila: cell autonomous requirement for Distal-less gene activity in limb development. *EMBO J.* **8**, 2045-2055.

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

Diaz-Benjumea, F. J., Cohen, B. and Cohen, S. M. (1994). Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. *Nature* **372**, 175-179.

Duncan, D. M., Burgess, E. A. and Duncan, I. (1998). Control of distal antennal identity and tarsal development in *Drosophila* by *spineless-aristapedia*, a homolog of the mammalian dioxin receptor. *Genes Dev.* **12**, 1290-1303.

Fleming, R. J., Gu, Y. and Hukriede, N. A. (1997). *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.

Fristrom, D. and Chihara, C. (1978). The mechanism of evagination of imaginal discs of Drosophila melanogaster. V. Evagination of disc fragments. *Dev. Biol.* **66**, 564-570.

Godt, D., Couderc, J.-L., Cramton, S. E. and Laski, F. A. (1993). Pattern formation in the limbs of *Drosophila*: *bric à brac* is expressed in both a gradient and a wave-like pattern and is required for specification and proper segmentation of the tarsus. *Development* **119**, 799-812.

Gorfinkiel, N., Morata, G. and Guerrero, I. (1997). The homeobox gene *Distal-less* induces ventral appendage development in *Drosophila*. *Genes Dev.* **11**, 2259-2271.

Gossler, A. and Hrabe de Angelis, M. (1998). Somitogenesis. *Curr. Top. Dev. Biol.* **38**, 225-287.

Goto, S. and Hayashi, S. (1999). Proximal to distal cell communication in the Drosophila leg provides a basis for an intercalary mechanism of limb patterning. *Development* **126**, 3407-3413.

Held, L. I. J., Heup, M. A., Sappington, M. and Peters, S. D. (1994). Interactions of *decapentaplegic*, *wingless*, and *Distal-less* in the *Drosophila* leg. *Roux's Arch. Dev. Biol.* **203**, 310-319.

Irvine, K. D. and Vogt, T. F. (1997). Dorsal-ventral signaling in limb development. *Curr. Opin. Cell Biol.* **9**, 867-876.

Irvine, K. D. and Rauskolb, C. (2001). Boundaries in development: formation and function. *Annu. Rev. Cell Dev. Biol.* **17**, 189-214.

Jockusch, E. L., Nulsen, C., Newfeld, S. J. and Nagy, L. M. (2000). Leg development in flies versus grasshoppers: differences in *dpp* expression do not lead to differences in the expression of downstream components of the leg patterning pathway. *Development* **127**, 1617-1626.

Kornberg, T. B. and Tabata, T. (1993). Segmentation of the Drosophila embryo. *Curr. Opin. Genet. Dev.* **3**, 585-594.

Kurant, E., Pai, C., Sharf, R., Halachmi, N., Sun, Y. H. and Salzberg, A. (1998). *dorsotonals/homothorax*, the Drosophila homologue of *meis1*, interacts with *extradenticle* in patterning the embryonic PNS. *Development* **125**, 1037-1048.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from Drosophila? *Cell* **85**, 951-961.

Lecuit, T. and Cohen, S. M. (1997). Proximal-distal axis formation in the *Drosophila* leg. *Nature* **388**, 139-145.

Mardon, G., Solomon, N. M. and Rubin, G. M. (1994). *dachshund* encodes

a nuclear protein required for normal eye and leg development in *Drosophila*. *Development* **120**, 3473-3486.

McGrew, M. J. and Pourquie, O. (1998). Somitogenesis: segmenting a vertebrate. *Curr. Opin. Genet. Dev.* **8**, 487-493.

Milan, M. and Cohen, S. M. (2000). Subdividing cell populations in the developing limbs of *Drosophila*: do wing veins and leg segments define units of growth control? *Dev. Biol.* **217**, 1-9.

Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al. (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-375.

Pai, C. Y., Kuo, T. S., Jaw, T. J., Kurant, E., Chen, C. T., Bessarab, D. A., Salzberg, A. and Sun, Y. H. (1998). The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, extradenticle, and suppresses eye development in Drosophila. *Genes Dev.* **12**, 435-446.

Panganiban, G., Nagy, L. and Carroll, S. B. (1994). The role of the *Distal-less* gene in the development and evolution of insect limbs. *Curr. Biol.* **4**, 671-675.

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 signaling in the *Drosophila* eye. *Science* **281**, 2031-2034.

Pick, L. (1998). Segmentation: painting stripes from flies to vertebrates. *Dev. Genet.* **23**, 1-10.

Rauskolb, C. and Irvine, K. D. (1999). Notch-mediated segmentation and growth control of the *Drosophila* leg. *Dev. Biol.* **210**, 339-350.

Rauskolb, C., Correia, T. and Irvine, K. D. (1999). Fringe-dependent separation of dorsal and ventral cells in the *Drosophila* wing. *Nature* **401**, 476-480.

Schubiger, G. (1971). Regeneration, duplication and transdetermination in fragments of the leg disc of *Drosophila melanogaster*. *Dev. Biol.* **26**, 277-295.

Schubiger, G. (1974). Acquisition of differentiative competence in the imaginal leg of *Drosophila*. *Wilhem Roux Archiv. EntwMech. Org.* **174**, 303-311.

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

Shen, W. and Mardon, G. (1997). Ectopic eye development in *Drosophila* induced by directed *dachshund* expression. *Development* **124**, 45-52.

Small, S. and Levine, M. (1991). The initiation of pair-rule stripes in the Drosophila blastoderm. *Curr. Opin. Genet. Dev.* **1**, 255-260.

Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. *Cell* **72**, 527-540.

Thomas, U., Speicher, S. A. and Knust, E. (1991). The Drosophila gene Serrate encodes an EGF-like transmembrane protein with a complex expression pattern in embryos and wing discs. *Development* **111**, 749-761.

Wu, J. and Cohen, S. M. (1999). Proximodistal axis formation in the *Drosophila* leg: subdivision into proximal and distal domains by Homothorax and Distal-less. *Development* **126**, 109-117.

Wu, J. and Cohen, S. M. (2000). Proximal distal axis formation in the Drosophila leg: distinct functions of Teashirt and Homothorax in the proximal leg. *Mech. Dev.* **94**, 47-56.