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Proximo-distal development in the legs of *Drosophila*

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ABSTRACT The appendages of insects and vertebrates develop as extensions of the body wall. During development, a proximo-distal axis for growth and patterning is created in each appendage, in order to specify appendage length and allocation of pattern elements like joints and sensory organs. Here we use the expression of molecular markers to examine how PD development takes place in the legs of the fruit-fly *Drosophila melanogaster*. The data suggest a process of regionalization and progressive subdivision of an anlage similar to both insect embryonic segmentation and vertebrate somitogenesis.

KEY WORDS: *appendages, legs, limbs, development, Drosophila*

Introduction

The appendages of the fly, just like the limbs of vertebrates, develop as extensions out of the embryonic flank. These extensions grow along an axis known as proximo-distal (PD), as it runs from proximal, or close to the body, to distal, further away from it. The development of such outgrowths needs to be controlled by the genome to reach the adequate appendage length; however appendages are more than just outgrowths of the body wall. Pattern formation also has to be specified along the PD axis as specific sensory organs, muscle attachments and joints have to be allocated in precise positions along the appendage. Thus, a PD axis for growth and patterning is created anew in each developing appendage, and the existence of such an axis is the basic developmental difference between the appendages and the rest of the body. This new PD axis is created using dorso-ventral (DV) and anterior-posterior (AP) positional cues, which the developing appendage often shares with other organs. Yet PD patterning uses further batteries of gene products which are expressed in particular PD domains of the developing appendage to stimulate cell division, allocation of cell fates, and eventually morphogenesis. PD domains of gene expression appear as caps or rings in the developing limbs of vertebrates; in *Drosophila*, PD domains appear as circles or rings in the imaginal discs (see below).

The most evident sign of PD organization in the legs of *Drosophila* are the joints and the so-called leg segments in between them (Fig. 1a). The leg segments and the joints span precise PD positions along the leg. There are 9 segments in the leg, all of which (except tarsal segments 2-4) have different lengths and differentiate specific pattern elements (Bryant, 1978). PD organization is also apparent in the undifferentiated appendages of the fly. These presumptive tissues form flat, quasi-circular epithelial sacs called imaginal discs. The imaginal discs look like folded and flattened versions of the final appendages (Fig. 1b,c) until at the end of imaginal development, the disc telescopes-out to differentiate as the final leg (reviewed in Fristrom and Fristrom, 1993). The pattern of folds in the discs just before evagination has helped to define "fate maps" that show how the different parts of the adult leg are represented in such mature discs (Bryant, 1978). In particular, different PD domains seen in the adult leg are determined in concentric rings of cells in the disc (see Fig. 1). Each leg disc before evagination consists of about 15,000 cells, but leg development has proceeded throughout 4 days of larval life starting from a small cluster of no more than 30 embryonic cells. These cells become mitotically quiescent and segregated from the rest of the embryo at about 8 h after egg laying (8 h AEL) (reviewed in Couso and Gonzalez-Gaitan, 1993). Invagination and formation of the imaginal disc is not accomplished until the beginning of the second larval instar (48 h AEL), when cell divisions are resumed (Madhavan and Schneiderman, 1977). The discs then grow by cell division for the next three days (48-120 h AEL, second and third instar) but there are no fate maps for the relatively featureless early discs, which lack the folds which help fate mapping of mature discs. Finally, the mature late third instar discs evaginate and differentiate during metamorphosis in the pupa to form the legs proper.

It is not possible to ascertain how PD development takes place by simple observation of the growing discs. Here we use the expression of molecular markers to address this question. The evidence suggests a process not unlike other processes of regionalization and progressive subdivision of an anlage, like embryonic segmentation. Since it is becoming apparent that organisms as different as insects and vertebrates may utilize a conserved set of genes and genetic interactions to generate limb pattern (reviewed in Perrimon, 1995; Shubin *et al.,* 1997), the contributions of this research in *Drosophila* could be of wider significance.

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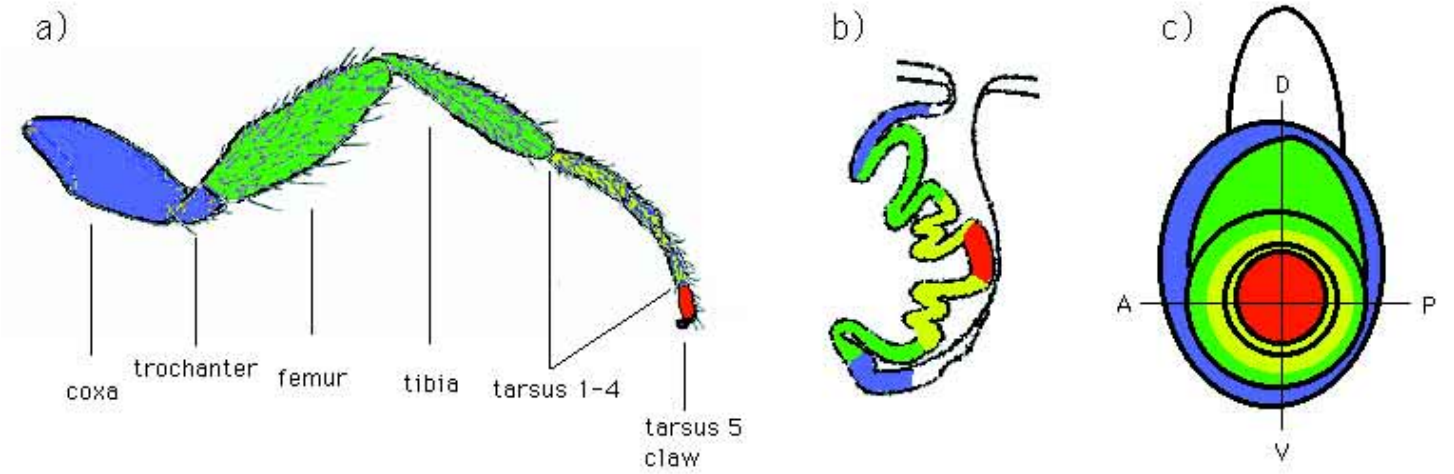

Fig. 1. Fate map of the imaginal leg disc of *Drosophila*. (a) *The fly leg is divided into 9 segments (coxa, trochanter, femur, tibia, and five tarsal segments), plus the claw, by joints located at precise positions along the proximo-distal (PD) axis.* **(b)** *Lateral view of a section through a leg imaginal disc just before evagination. The disc looks as if the final leg were squashed from the tip. The presumptive regions for the different PD leg segments can be identified by the stereotyped pattern of folds.* **(c)** *Frontal view of the same leg imaginal disc. The pattern of folds can still be appreciated, although in this view some portions of the disc cannot be seen as some folded tissues lie on top of each other (compare with B). The disc shows the PD organization as a series of concentric circles and rings. The presumptive regions for the most distal part of the leg (5th tarsal segment and claw) are located in the centre of the disc (red). More medial presumptive regions appear as circumferences around the centre (yellow, green), and the most proximal regions are located at the periphery of the disc (blue). The anterior-posterior (A-P) and dorsal-ventral (D-V) axes of the disc intersect at the centre, at the presumptive tip of the leg. Notice how these AP and DV axes divide the disc into 4 sectors.*

PD gene expression during leg development

In *Drosophila*, there are several identified genes (thereafter called PD genes) which are expressed in concentric circles or rings in the late third instar discs and whose mutant phenotypes lack specific PD regions of the adult leg. These regions affected in the respective mutants illustrate the realm of action of each gene throughout development. Provided that no major discrepancies are found between mutant phenotypes and late third instar domains of expression, we can then use the expression of PD genes as fate markers to visualize how and when different PD fates are created in the leg throughout development.

The first PD gene to be expressed is *Distal-less (Dll)*. The homeobox-containing *Dll* protein is already present at about six hours of embryogenesis (stage 11) in clusters of cells in the positions where leg primordia will be identifiable later (Cohen *et al.,* 1989). *Dll* expression (Fig. 2A) is required early for the development of the vestigial larval legs of *Drosophila*, the Keilin's organs (Vachon *et al.,* 1992), and later for the development of the distal structures of the leg disc (Sunkel and Whittle, 1987; Cohen and Jurgens 1989). It has been shown that the allocation of *Dll* expression in the leg primordia depends on some of the positional cues that generate the DV and AP patterns of the larval epidermis, like *wg*, *dpp* and DER (Cohen *et al.,* 1993; Goto and Hayashi, 1997). Afterwards, *Dll* expression seems to become independent of *wg* and *dpp* (Lecuit and Cohen, 1997), probably through a self-maintenance mechanism (Castelli-Gair and Akam, 1995).

Dll expressing cells are recruited into the invaginating discs and are present in first and early second instar discs (24-48 h AEL) which have already segregated from larval tissues (unpublished observation and Fig. 2B). In late third instar discs the *Dll* expressing regions can be identified as distal tibia and tarsal segments 1 to 5 (Fig. 2D-E). These regions are absent in *Dll* mutants where *Dll* function has been removed during the third instar (Sunkel and Whittle, 1987). However, cells deprived of *Dll* function before second instar (48 h AEL) cannot give rise to trochanter, femur or proximal tibia either (Cohen and Jurgens, 1989; Cohen *et al.,* 1993). One possible interpretation of this discrepancy is that *Dll* may be transiently expressed and required in the presumptive regions of trochanter, femur and proximal tibia before 48 h AEL. Thereafter *Dll* expression would be confined to the presumptive regions of distal tibia and tarsus for the rest of development. Hypomorphic *Dll* mutant animals (Fig. 2F) illustrate these dynamic requirements by displaying abnormal trochanter, femur and tibia, and absent tarsi.

A second homeobox gene, *extradenticle (exd)*, is also expressed in the leg primordia in the embryo, but it is excluded from those cells expressing *Dll*. In the 48 h AEL disc *exd* can be seen expressed around the *Dll* domain (Gonzalez-Crespo and Morata, 1996). The abutting expression of these genes suggests that they may have an antagonistic relationship. In fact, lack of *exd* function eliminates proximal fates, whereas ectopic *exd* expression in the *Dll* domain results in loss of distal cells (Gonzalez-Crespo and Morata, 1996). This suggests that the role of *exd* is to promote the growth of cells with proximal fates. Later in development, the expression of the gene *tsh* is activated in a ring coinciding with *exd* (Fig. 3A; Gonzalez-Crespo and Morata, 1996). Fate changes following lack of function of *tsh* and ectopic expression of *tsh* suggest that *tsh* is involved in defining proximal identity (Steve Kerridge, personal communication).

The nascent leg anlage is therefore divided initially into two domains with distinct PD identities. This division provides PD polarity to the anlage and is required to promote further PD development, because total removal of *Dll* function from embryogenesis prevents any distalization of the legs further than formation of the coxa (Cohen *et al.,* 1993).

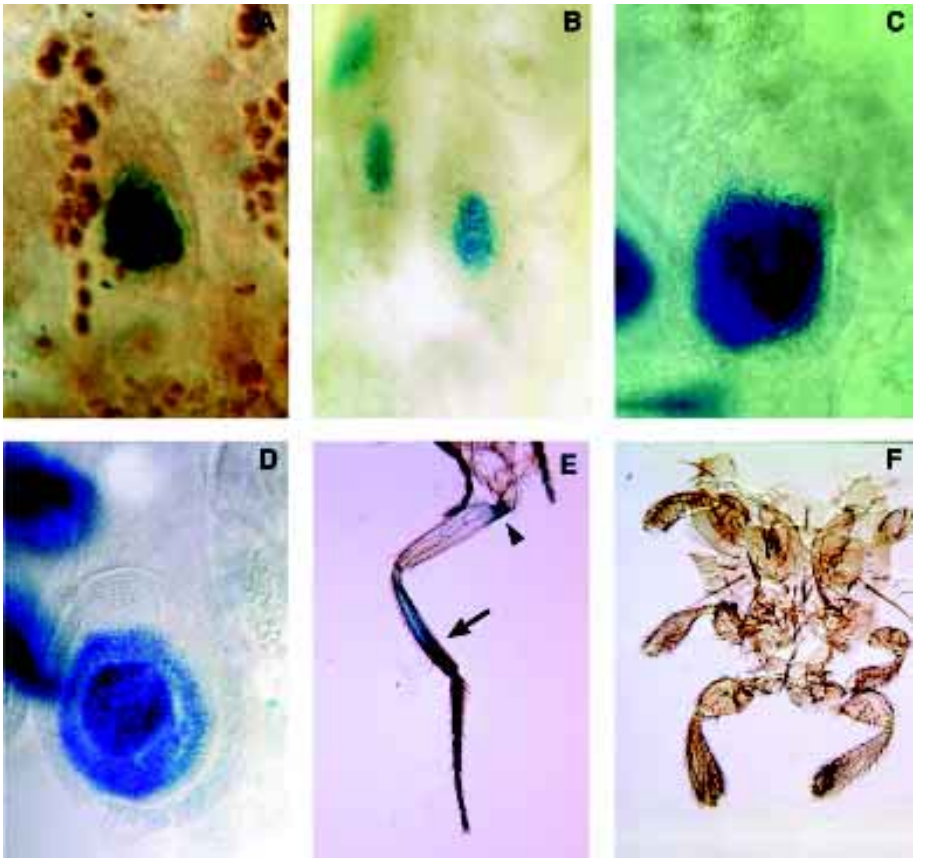

Fig. 2. Expression and function of the gene *Dll* along the PD axis. (A) *Dll expression (blue) in the leg imaginal primordia of an embryo at 10 h AEL. Dll expression has been monitored by revealing the pattern of lacZ enzymatic activity in a transgenic strain (Dll^{1092}) which carries a lacZ gene inserted in the Dll gene. The embryo has also been stained with an antibody against the cut protein (red) to identify sensory organs.* **(B)** *Dll^{1092} leg imaginal discs from a 72 h AEL larva showing Dll expression (blue) at the centre of the discs.* **(C)** *Dll expression (blue) in a mid-third instar larva (aprox. 96 h AEL).* **(D)** *Dll expression in a late third instar disc (aprox. 120 h AEL). This plane of focus shows the expression in the presumptive regions for the distal tibia and the tarsi. The proximal ring of expression in the trochanter lies underneath this plane of focus and cannot be seen (see Fig. 1b,c).* **(E)** *Leg from a Dll^{1092} adult stained for lacZ activity (blue). Expression can be seen to extend from the tip of the leg (at the bottom of the Figure) to the middle of the tibia (arrow). The proximal expression in the trochanter (arrowhead) can be seen.* **(F)** *Legs from a Dll^{3} mutant fly. Lack of function of Dll produces a leg truncated at the tibia. The remaining tibia, femur and trochanter are shorter than wild-type and deformed (compare with E).*

The *dachshund (dac)* gene encodes a novel nuclear protein required for the morphogenesis of femur, tibia and the first three tarsal segments (Mardon *et al.,* 1994). *dac* expression is absent in the leg primordia in embryogenesis but in the second instar, during the third day of development (48-72 h AEL), *dac* expression is found in a ring abutting the circle of *Dll* expression (Mardon *et al.,* 1994; Lecuit and Cohen, 1997). It can be inferred that by second instar, medial PD identities have been intercalated in the leg disc in between the proximal region defined by *exd* and the more distal one defined by *Dll*. Later in development, during the third instar, the expression of *dac* and *Dll* becomes overlapping in the presumptive tibia and tarsi 1-3 (Fig. 3B; Lecuit and Cohen, 1997). It is interesting to note that *dac* expression is activated in second instar in the regions that seem to lose *Dll* expression at that time (see above). Although it has been suggested that the ring of *dac* expression is directly set up by the DV patterning proteins *Wg* and *Dpp* (Lecuit and Cohen 1997), it is possible that a repressory action by *Dll* is also involved (I. Galindo and J.P. Couso, unpublished observations).

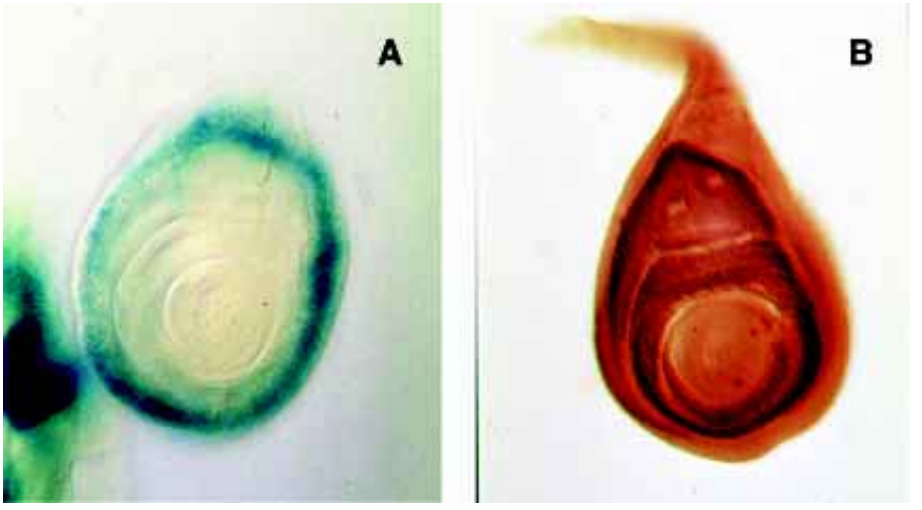

Fig. 3. Proximal and medial PD domains in the leg disc. (A) *Expression of the tsh gene (blue) is revealed as in Figure 2 in a late third instar (120 h AEL) leg disc from a tsh^{lacZ} larva. tsh is expressed in the most proximal presumptive regions of the disc, much like exd at this stage (Gonzalez-Crespo and Morata, 1996).* **(B)** *dac expression (red) in a dac^{lacZ} leg disc of a similar age as in (A). The pattern of expression has been revealed using antibodies against the protein produced by the lacZ gene inserted at the dac locus. dac is expressed at this stage in the presumptive regions of the femur, but also in the tibia and tarsal segments 1 to 3, where it overlaps Dll expression (compare with Fig. 2D).*

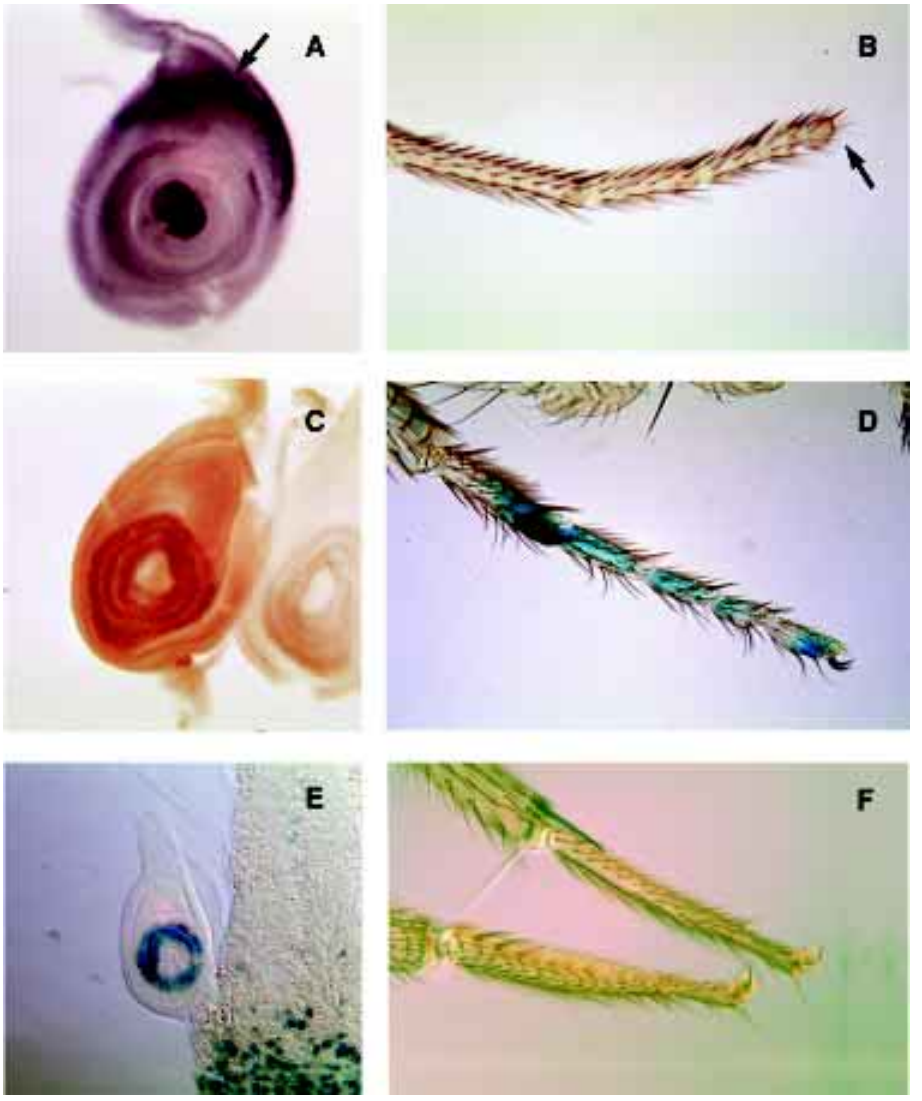

Fig. 4. Tarsal development during third instar (72-96 h AEL). (A) *al expression (purple), monitored with an antibody against the al protein (Campbell et al., 1993). al expression is seen at the centre of the disc, in the presumptive regions of the 5th tarsal segment and the claw (see B). Expression can also be found at the periphery of the disc (arrow) in the presumptive regions of the proximal body wall.* **(B)** *Detail of the tip of the leg from an al mutant fly (Dfal/al^{ice}). The 5th tarsal segment (arrow) is reduced and the claw is missing (compare with D). In addition to this, the sternopleural bristles in the body wall are lost in these mutants (not shown).* **(C)** *rn gene expression (revealed as in Fig. 3B) in a leg disc from a late third instar rn^{lacZ} larva. Expression is localized in the presumptive regions for tarsi 1 to 4. Note the modulation in rings of the labeling.* **(D)** *Leg from a rn^{lacZ} adult fly, stained for lacZ activity (blue). The modulation in rings seen in (C) appears as regions with more intense blue staining. Staining also appears near the claw (right).* **(E)** *Mid-third instar (96h AEL) leg disc from a rn^{lacZ} larva showing the initial activation of rn expression in a narrow ring (blue) around the regions which express al at this stage. Expression of bab is identical to that of rn throughout development.* **(F)** *Legs from a rn mutant fly (Dfdsx10/rn^{3}). The tarsal segments 2 to 4 are reduced and fused, with no joints formed between them. Comparison of the leg at the bottom of the Figure with the wild-type looking leg of (D) shows the overall reduction in size of the tarsal region. The rn mutant tarsal region looks like a wild-type tarsus 1 fused to a tarsus 5.*

At the beginning of the third instar, the expression of the homeobox gene *aristaless (al)* is added in the centre of the growing leg disc (unpublished observations; Campbell *et al.,* 1993). This activation has been shown to depend on *wg* and *dpp* function (Campbell *et al.,* 1993), although this effect could be indirect and mediated by *Dll* or another PD gene. In the late third instar *al* expression is found in the presumptive tip of the leg, and loss of *al* function eliminates the *al*-expressing areas of claw organ and part of the fifth tarsal segment (Fig. 4A,B). The onset of *al* expression around 72 h AEL can be taken to signify that the distal-most regions of the leg have been defined.

Shortly before mid-third instar (96 h AEL), the activation of *rotund (rn)* and *bric-a-brac (bab)* expression takes place around the *al* domain in a narrow ring which expands during the rest of development (Fig. 4 C-E). In the late third instar disc, *bab* and *rn* expression can be fate-mapped to the presumptive regions for tarsi 2 to 4, which are affected in the mutants (Fig. 4C,D,F; Agnel *et al.,* 1992; Godt *et al.,* 1993). It is tempting to speculate that expression of both genes is used to intercalate tarsal fates at the interface between *al* and non-*al* expressing cells. This intercalation would involve a cell communication process at such an interface. However *bab* encodes a Zn-finger protein (Godt *et al.,* 1993) and the molecular nature of the *rn* product is as yet unknown.

In the period from mid to late third instar, the final elements of the PD pattern are added to the disc. There seems to be a "second round" of PD patterning as *Dll* and *al* expression is activated in rings at the periphery of the disc. A late ring of *Dll* expression appears in the trochanter, where it contributes to trochanter patterning (Fig. 2E,F). *al* expression is activated in two proximal rings, one of them in the presumptive body wall or pleura regions where the lack of *al* function eliminates proximal pattern elements (Schneitz *et al.,* 1993). Finally, the *apterous* gene is expressed in a single ring in the 4th tarsal segment but no mutant phenotype has been associated with this expression (Cohen, 1993), maybe simply because a transformation of 4th tarsus into 3rd tarsus could pass unnoticed as the morphology of these two tarsal segments is identical.

The most remarkable event during this late period of leg development is the establishment of repeated patterns of gene expression. These are seen as a series of concentric rings at the presumptive joints, and appear in no obvious PD sequence during

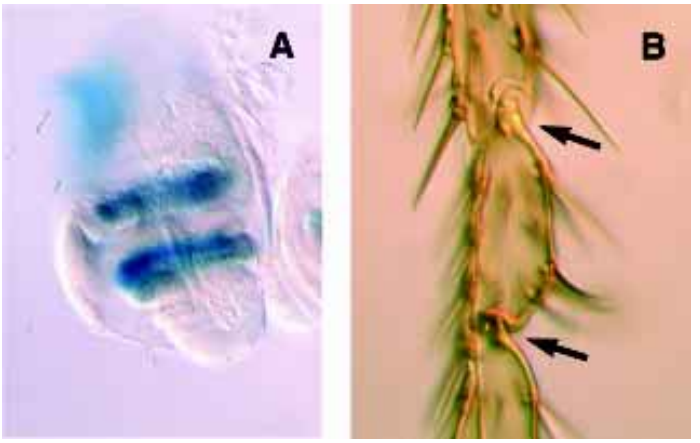

Fig. 5. Development of joints. (A) *Lateral view of a 124 h AEL leg imaginal disc which is beginning to evaginate, showing two repeated stripes of fj expression (blue; distal to the bottom). fj expression has been revealed in a* fj^{lacZ} *larva as in Figure 1 and is located around presumptive joint regions in the first tarsal segment and the tibia. Expression in other tarsal joints appears later in development.* **(B)** *Detail of the tarsi from a wild-type leg, showing the jointed articulations at the segment boundaries (arrows).*

late third instar and early pupa until each joint shows a ring (unpublished observations; Cohen, 1993). For example, the *four-jointed* gene *(fj)* gene starts to be expressed in presumptive joint areas (Fig. 5A,B) and has been shown to be required to form joints in early pupa (Villano and Katz, 1995). The *Notch (N)* and *Delta (Dl)* genes are also required for joint development from earlier than the onset of *fj* expression (Schellenbarger and Mohler, 1978; Parody and Muskavitch, 1993). In their milder phenotypes mutants for *fj*, *N* and *Dl* produce loss of joints, but stronger or earlier lack of function reduces the size of the leg segments as well. The intercalation of *fj* and other ring patterns at the leg segment boundaries indicates that the leg segments are determined by late third instar as distinct cell populations. Indeed, these rings could be activated using cues from existing PD domains. For example, *rn* and *bab* expression show a modulation in rings at the tarsal joints (Fig. 4C,D), and weak *rn*, *bab* and *Dll* phenotypes produce a loss of joints comparable to *fj*, *N* or *Dl* phenotypes. A possible mechanism for intercalation of rings at the interfaces of different PD populations is discussed below.

Mechanisms for PD pattern formation

The sequence of events in PD development (summarized in Fig. 6) suggests a broad similarity with other patterning processes in *Drosophila*. Firstly polarization of the leg disc with the establishment of a PD axis takes place. Refinement, subdivision and further growth of these regions follow, with new regions being specified in no obvious PD sequence. For example, the leg is extended in the early third instar by the addition of *al* expression to create the fifth tarsal segment and the claw, but the other tarsi are intercalated afterwards by the onset of *rn-bab* in a ring domain abutting that of *al*. Finally, during the late third instar and early pupa these regions grow and develop stereotyped metameric patterns (tarsal joints), by the activation of genes expressed in rings repeated at each joint. How are all these different cell fates generated along the PD axis?

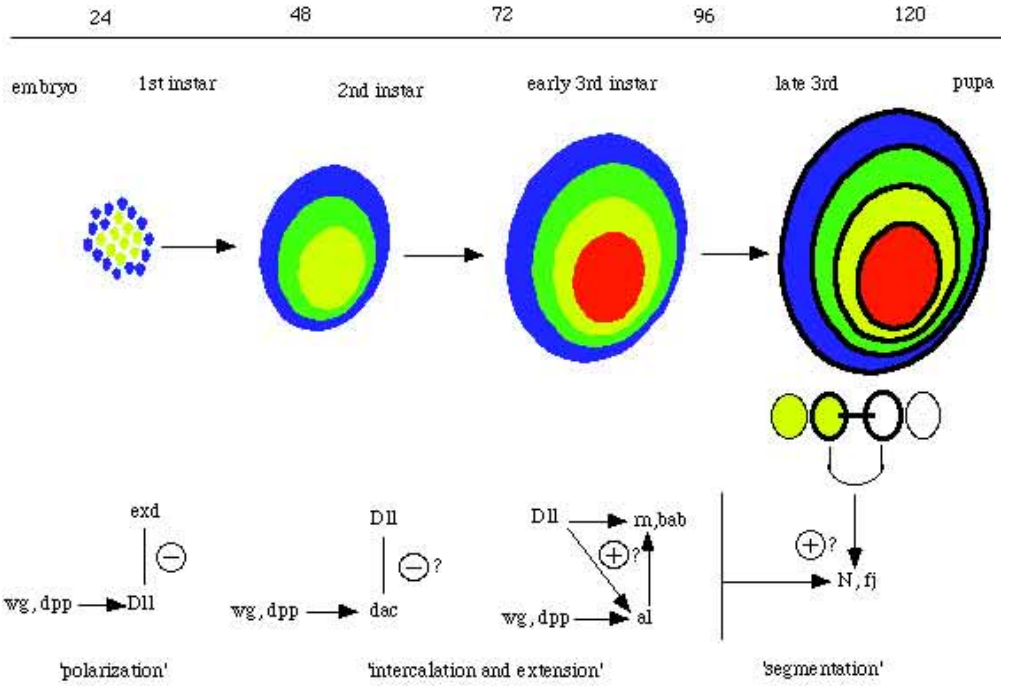

Fig. 6. Summary of PD development in the leg discs. *The upper bar shows time of development as hours after egg laying (h AEL).The first PD specification takes place within the leg primordia during the first 2 days of development (embryo and first larval instar, 12-48 h AEL). Some cells of the primordia express Dll (yellow) whereas the rest express exd (blue), and this provides the polarization of the leg disc with the establishment of PD axis. During second instar, dac expression (green) is intercalated in a ring of cells around the central circle of Dll expression by the combined action of dpp and wg, and possibly also repression by Dll. In this way, the disc is now endowed with rough proximal, medial and distal identities. During the 4th day, or early third instar, (72-96 h AEL), further subdivision and growth of these regions takes place, so for example, the tarsi are determined in the distal cells of the leg disc by the onset of rn-bab (yellow) and al (red) expressing regions. al may be activated by the effect of wg and dpp upon Dll expressing cells whereas rn and bab may be set up by interaction of al with neighboring cells. Finally, during the fifth and sixth days (96-132 AEL, late larva and early pupa) these regions grow and develop stereotyped metameric patterns (tarsal joints), by the activity of genes in repeated rings at each joint (fj, Dl and N; black rings). These genes may be activated at the interfaces of cells with different PD identities, by a cell communication mechanism similar to that at work in the DV boundary in the wing.*

It has been known for some time that insect legs and vertebrate limbs are organized using two systems of positional co-ordinates, one circumferential around the leg and another PD along the leg (French *et al.,* 1976). These inferences have been given a molecular meaning in recent years. Genes *(wg, hh, dpp)* expressed in sectors of the disc determine the circumferential pattern around the leg, promoting dorsal, ventral, anterior and posterior fates (Couso *et al.,* 1993; Held 1993; Brook and Cohen 1996; Sanchez-Herrero *et al.,* 1996; Theisen *et al.,* 1996). It has been shown that these genes are also required to drive distalization, and it has been proposed that the combined action of the *wg* and *dpp* secreted proteins near the centre of the leg disc works as a morphogenetic gradient of positional information which activates the expression of PD genes like *Dll* and *al* (Campbell *et al.,* 1993; Diaz-Benjumea *et al.,* 1994; reviewed in Held 1995).

A model for distalization (Meinhardt, 1986; Campbell *et al.,* 1993) has been elaborated to accommodate these observations. It proposes only an initial input from *wg* and *dpp* to set up the distal-most regions in the early leg disc, which the model assumes to be hitherto composed only of proximal cells. Then, intercalation between these distal-most and proximal-most regions would give rise to the rest of the leg. This intercalation would depend on cell interactions, driven by PD genes, to generate new PD positional values and drive limb growth. However, in the face of the observations presented here, it seems that this model is not entirely correct. As we have mentioned before, leg PD values seem to be intercalated or added in no special PD sequence; and several studies suggest that *wg*, *hh* and *dpp* are required continuously, and not only initially, for distalization (Couso *et al.,* 1993; Bassler and Struhl, 1994; Lecuit and Cohen, 1997).

The opposite view would question whether there is an active PD patterning process at all, working independently of circumferential patterning genes like *dpp* or *wg*. The PD genes, very much like homeotic/*Hox* genes (reviewed in McGinnis and Krumlauf, 1992), could be just a group of transcription factors that record and interpret positional information generated by others, in our case the circumferential patterning genes *wg*, *dpp* and *hh*. Although it is difficult to imagine that the combined diffusion of *wg* and *dpp* proteins can define all the PD regions of the leg, this mechanism is formally possible (Lecuit and Cohen, 1997). It is relevant to note that all the early PD genes which have been molecularly characterized *(Dll, exd, dac, al, bab)* seem to encode transcription factors. Interestingly, the *Hox* genes themselves are implicated in PD specification in vertebrate limbs (McGinnis and Krumlauf, 1992).

These data might cast doubt on the existence of an active PD patterning process, but there is more evidence to be considered. Firstly, some findings suggest the existence of cell interactions which allow the cells to recognize their PD positional value and those of their neighbors so as to act in consequence: it has been observed that imaginal leg discs regenerate their PD axis following experimental manipulation (French *et al.,* 1976), and also that imaginal leg cells sort themselves out according to their PD fate, either in cell re-aggregates (Garcia-Bellido, 1966) or in *Dll* mosaic animals (Cohen and Jurgens, 1989). Secondly, at the genetic level, not all PD patterns of expression are dependent on *wg* and *dpp*. *exd* expression does not require *hh* function (and, by extension, neither *wg* nor *dpp*; Gonzalez-Crespo and Morata, 1996), whereas *al* expression, although ultimately dependent on *wg* and *dpp* function, has not been shown to be directly activated by them. *dac* and *Dll* have been shown to be most probably directly activated by *wg* and *dpp* in initially abutting domains (Lecuit and Cohen, 1997), but later on these domains become overlapping, suggesting a transient repressory relationship. This transition has been attributed to the combined effects of *Dll* self-maintenance and distal growth (Lecuit and Cohen, 1997). However, cell lineage tracing experiments show that the allocation of PD fates to cells does not occur by a lineage-based mechanism, and that growth occurs throughout the whole leg and not just at the distal end (Bryant and Schneiderman, 1969).

Finally, if the PD pattern just read the information provided by *wg* and *dpp*, the PD genes should not interact with each other. However, our preliminary results show that the expression of PD genes is not independent of each other.

We therefore favor a scenario in which after the initial polarization of the anlage by circumferential genes, the integration of inputs from circumferential and PD genes intercalates and extends the rest of the PD fates (Fig. 6). It is possible to envisage a situation comparable to the eye, where an input stimulates a generalized "neural" development, while the precise nature of the neuron differentiated depends on the developmental history of the anlage and on which fates have been determined already. In the leg, *wg* and *dpp* could provide a constant distalization stimulus which would be modulated by the PD patterning genes already in place. The early activity of *wg* and *dpp* would result in activation of *Dll* in the embryo; but then repression by *Dll* would restrict *wg*/*dpp*-mediated activation of *dac* to non-*Dll* expressing cells in second instar. Yet later in third instar, the prolonged presence of *Dll*, *wg* and *dpp* at the centre of the disc would combine to produce the activation of *al*. This picture would be like many other patterning cascades; some "primary" genes *(Dll*, *dac* and *al)* would translate positional information from a "seeding" patterning system *(hh, wg, dpp)* to initiate a new patterning process; then, these "primary" genes would activate and interact with others (*exd*, *rn*, *bab*, etc.) to refine the pattern independently of the 'seeding' patterning cascade.

One important instance of PD leg patterning does require cell signaling and interaction between cells with different PD identities: joint development. The *Dl* and *N* proteins work as ligand and receptor during several cell fate choices during fly development (reviewed in Muskavitch, 1994). The similarity of their mutant phenotypes suggests that they might also be functionally related during joint development. *N* signaling is usually associated with the determination of single cells or with the establishment of organizing boundaries between distinct cell populations. It is difficult to see that joint placement requires single cell precision, since the joints are multicellular structures (Fristrom and Fristrom, 1993). However, it is possible that *N* signaling is establishing a local organizing centre from which further patterning and growth of the tarsi and other leg segments is directed, since in extreme N mutant conditions not only the joints but whole tarsal segments are lost. Rather like in the DV boundary in the wing (Couso *et al.,* 1995; Kim *et al.,* 1995), where *N* signaling drives wing growth and eventually produces a multicellular pattern feature, the wing margin (Couso *et al.,* 1994), formation of the leg joints may involve many cells and drive the final growth of the leg segments. In this regard it is interesting to note that the *fj* protein displays similar characteristics to the *hh* protein, suggesting that it might be a short-range signaling molecule, as indeed its phenotypes in mosaics suggest (Villano

and Katz, 1995). It follows that, just like in the DV boundary in the wing, a boundary between different cell populations can be used to set up a patterning centre which then drives the development of the neighboring regions. In the legs these organizing boundaries would be repeated through the anlage, and so they could be better compared to embryonic segment boundaries (reviewed in Martinez Arias, 1993).

Segmentation of legs

The onset of repeated and concentric rings of expression in the leg during the third instar might indicate that, just like with the onset of stripe patterns in the embryo, a continuous anlage has been divided into homologous and independent units using non-periodic references. Leg segments may be true segments in cockroaches at least , as they behave as fields or units of regeneration which regenerate inside but not outside pattern elements (reviewed in French *et al.,* 1976). Indeed, *N-Dl* signaling is used in vertebrates to generate the pattern of somites, showing that N signaling can be used to subdivide an anlage into metameric units (Conlon *et al.,* 1995; Hrabe de Angelis *et al.,* 1997). Much like embryonic segmentation clearly provides a useful device whose capacities have been exploited to the full during annelid and arthropod evolution, leg segmentation allows the formation of stereotyped articulations or joints, which confers a much greater degree of functionality and flexibility to the arthropod appendage than would a solid outgrowth. Furthermore, during arthropod evolution the PD morphology of the appendages has been repeatedly altered and many different outcomes seem to spring from an ability to modify independently each segment of the appendage.

Embryonic segmentation in *Drosophila* takes place in a syncytium which does not proliferate, a situation in which in effect the anlage is grown first and specified later. In contrast, we have seen that the legs appear to be progressively specified as they grow. Leg segmentation appears therefore more similar to the process of embryonic segmentation in more primitive insects like locust, grasshopper and *Tribolium*. In these, segmentation takes place in a growing, proliferating cellular anlage, where segments grow and get added to the embryo (reviewed in Tautz and Sommer, 1995). Development follows a broad anterior to posterior sequence, where new segments are added at the posterior terminus of the embryo. However, new segments may also appear anterior to the last one, that is intercalated between existing segments. Just like with leg segments, we find both extension and intercalation at work in these embryos. Thus, although the molecules implicated are different, the logic and the developmental problem for the embryo of a primitive insect may be the same as for any insect leg. It would be interesting to study the involvement of more cell communication proteins in segmentation in primitive insects. Further than conservation of gene batteries, we may find the same molecular logic at work as in insect legs and vertebrate somites.

Here we have presented a preliminary scenario for PD development. However, many aspects presented here are yet to be tested or proven at the molecular level. Furthermore, there are many gaps in our understanding of the processes outlined, especially in the activation of gene expression patterns. By no means have we identified all the genes controlling the growth, identity and morphogenesis of each part of the leg. We have taken here patterns of gene expression to indicate that presumptive regions of the leg are defined, but it does not follow that these genes themselves are responsible for the determination of those leg parts. Furthermore, in some regions more than one gene may be required to control cell growth and identity, as in the cases of *bab* and *rn* in the tarsi, or *exd* and *tsh* in the proximal leg, thus increasing the list of genes yet to be identified. To understand the molecular and genetic basis of distalization will require us to understand the logic of the genetic regulatory relationships between the genes at work in the process; their assortment into functional hierarchies, and the molecular mechanisms which underlie all of those. If an active PD patterning system exists, more PD genes encoding cell signaling and transduction proteins will have to be identified, and gene regulation processes based on cell communication discovered.

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