Flexible joints separate the rigid sections of the insect leg, allowing them to move. In Drosophila, the initial patterning of these joints is apparent in the larval imaginal discs from which the adult legs will develop. Here, we describe the later patterning and morphogenesis of the joints, which occur after pupariation (AP). In the tibial/tarsal joint, the apodeme insertion site provides a fixed marker for the boundary between proximal and distal joint territories (the P/D boundary). Cells on either side of this boundary behave differently during morphogenesis. Morphogenesis begins with the apical constriction of distal joint cells, about 24 h AP. Distal cells then become columnar, causing distal tissue nearest the P/D boundary to fold into the leg. In the last stage of joint morphogenesis, the proximal joint cells closest to the P/D boundary align and elongate to form a “palisade” (a row of columnar cells) over the distal joint cells. The proximal and distal joint territories are characterised by the differential organisation of cytoskeletal and extracellular matrix proteins, and by the differential expression of enhancer trap lines and other gene markers. These markers also define a number of more localised territories within the pupal joint.

Key Words: arthropod limb; leg imaginal discs; metamorphosis; morphogenesis; cell shape; joint territories; tibia; tarsus; SEM; GAL4 enhancer trap; plasmid rescue.

INTRODUCTION

A distinguishing feature of arthropods is the possession of jointed limbs. Insects and other arthropods have hard external skeletons. To move, this armor must have pliable parts that are stayed against more rigid parts. The adult leg joint possesses both of these elements. Flexible intersegmental membrane, made of unsclerotised cuticle, allows movement between leg segments. Rigid points of articulation, or condyles, between the two shafts of opposing leg segments restrict the angle of joint flexure (Snodgrass, 1935). Together, these structures enable the leg to bend in a controlled manner.

Leg joints first become apparent as rings of patterning gene expression in the larval imaginal discs. However, like many adult structures, leg joints do not differentiate until pupal development. Joint morphogenesis begins at 24 h after pupariation (AP) when cells in the tarsal joints constrict (Fristrom and Fristrom, 1993). This separation between when legs are patterned and when they differentiate makes leg joints an excellent system to study how connections are made between cell identity and morphogenesis.

The anteroposterior and dorsoventral patterning systems of the body segments interact to trigger initial proximodistal patterning of the leg (Cohen, 1993). By the second larval instar, the secreted growth factors Decapentaplagic (Dpp) and Wingless (Wg) are expressed in dorsal and ventral sectors, respectively, in the developing leg disc. At the tip of the leg, where the pretarsus will form, the expression of these two proteins overlaps and this interaction maintains Distalless (Dll) expression (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Dll induction leads to a patterning cascade whereby genes are activated or repressed in broad regions along the length of the limb. The most proximal parts express Homothorax, medial regions such as presumptive femur, tibia, and first tarsal segments express Dachshund, and distal parts express Dll (Cohen and Jurgens, 1989; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Dll induction leads to a patterning cascade whereby genes are activated or repressed in broad regions along the length of the limb. The model system for larval joint development is Drosophila, which has a well-characterised pattern of gene expression and morphogenesis.
rings in the leg discs (Couso and Bishop, 1998; de Celis et al., 1995, 1998; Rauskolb and Irvine, 1999; Bishop et al., 1999).

The specification of joint tissue in the leg disc requires the function of the Notch signaling pathway (de Celis et al., 1995, 1998, Rauskolb and Irvine, 1999, Bishop et al., 1999). Notch and its ligands are expressed in a pattern of complementary rings where Notch is active on the distalmost side of the joint and its ligands are expressed proximally (de Celis et al., 1998, Rauskolb and Irvine, 1999, Bishop et al., 1999). If any one of the Notch signaling proteins is removed from joint cells, joints fail to form. Conversely, ectopic expression of Notch ligands (such as Delta or Serrate) or ectopic activation of the Notch signaling cascade can produce ectopic joint tissue (de Celis et al., 1998, Rauskolb and Irvine, 1999, Bishop et al., 1999).

Although the location of each leg joint is already established by the time the animal pupariates, the complexity of the adult structure suggests that additional patterning steps are required. Notch and its ligands remain expressed in the joint cells throughout leg development (de Celis et al., 1998), so it is likely that these molecules take on new roles as joint development progresses. However, neither the process of joint morphogenesis nor the expression of other markers that appear later in joint development have been investigated.

In this study, we describe the cell shape changes and changes in the distribution of filamentous actin and two extracellular matrix proteins that occur during joint differentiation. We identify distinct populations of cells within the developing joint by correlating these cell shape changes with the expression patterns of joint markers. Using a set of new enhancer trap insertions as well as four previously described joint markers, we show that the expression of many genes involved in joint development changes significantly between the wandering L3 and the pupal stages, when joints actually form. Although this study focuses primarily on the tibial/tarsal joint, we make comparisons between this joint and similar events occurring in the tarsal joints.

**MATERIALS AND METHODS**

**Fly Stocks**

To mark posterior territories in the differentiating joint, we used en-gal4-lacZ (enlacZ), a reporter construct that drives the expression of β-galactosidase (β-gal) in the posterior cells of each segment. Other previously described enhancer trap lines used to examine joint territories were odd-skipped lacZ and disconnected lacZ (Bishop et al., 1999; Rauskolb and Irvine, 1999). Two sets of novel P{GawB} insertions (GAL4 lines) were also used, one generated for the purposes of this study (see Enhancer Trapping below) and the other isolated in Dr. O’Kane’s laboratory. These GAL4 lines were crossed to one of three stocks: (1) a stock carrying UAS-GFP on the third chromosome (D1–Ba’ar11 UAS-GFP 65/167; a gift from Dr. Andrea Brand), (2) a stock carrying enlacZ on the second chromosome and UAS-GFP on the third (w; enlacZ/Cyo; UAS-GFP), or (3) a stock carrying wingless lacZ (wg lacZ) (which marks a ventral domain of the leg) on the second chromosome and UAS-GFP on the third (w; Sco/Cyo wg lacZ; UAS-GFP).

**Microscopy**

We used several techniques to describe joints: (1) scanning electron microscopy (SEM) of adult and pupal joints, (2) imaging of developing joints using Nomarski optics, and (3) confocal microscopy to examine cell shape changes and the expression of joint markers. In all cases, pupae were collected either every 2 h (0–2 h AP) or every 4 hours (0–4 h AP) and allowed to develop at 25°C until they reached the desired stage.

**SEM of adult and pupal legs.** To prepare adult legs for SEM, the legs were removed and fixed in 70% ethanol/30% glycerol. Legs were dehydrated by washing them in 70% ethanol, 95% ethanol, and 100% ethanol, critical point dried using CO2 as the transitional fluid, mounted onto aluminum stubs using adhesive strips, and coated with gold in a Polaron sputter coater ES5000.

For SEM of pupal legs, pupae were dissected out of their pupal cases in 0.05M Pipes (piperazine-N,N’-bis(2-ethanesulfonic acid); Sigma). Their thoraces, along with the legs and wings, were removed and fixed overnight in 0.75% glutaraldehyde in 0.05M Pipes. The following day, the pupal cuticle was removed from legs by using fine forceps, the wings were torn off, and the legs were separated and fixed overnight in 3% glutaraldehyde in 0.05M Pipes. Legs were washed (0.05M PIPES, 4 times for 15 min each wash) and treated with 1% osmium tetroxide for 1 h. After rinsing the legs with water (4 times for 15 min), the legs were dehydrated and mounted as above.

Images for both adult and pupal legs were collected either on film from a Philips XL30 FEG Scanning Electron Microscope 505 or digitally from a Philips XL30 FEG Scanning Electron Microscope.

**Nomarski imaging of pupal legs.** Pupal legs were dissected in phosphate-buffered saline (PBS) and fixed overnight in 2.5% glutaraldehyde in PBS. The pupal cuticle was peeled off the legs; then the legs were separated and finally mounted in 100% glycerol. Images were captured using a Zeiss Axioshot Microscope with Nomarski optics either on film or with a Hamamatsu digital camera controlled by Improvision OpenLab software.

**Antibody staining of pupal legs.** Thoraces were dissected from pupae in PBS and fixed in 4% paraformaldehyde in PBS for between 20 min and 24 h (depending on the primary antibody used; details on request). After rinsing away the fixative, the pupal cuticle was removed so that the antibody could access the tissue. Thoraces were then washed with PBT (PBS and 0.3% Triton X-100; 4 times for 15 min) and blocked in PBT and 3% goat serum for 30 min to minimize background staining. Legs were incubated in primary antibody diluted in PBT and 3% goat serum overnight at 4°C or at room temperature (RT). Primary antibodies used included rabbit anti-β-gal (commercial, 1:1000), rabbit anti-Laminin (Fessler et al., 1987, 1:500), mouse monoclonal anti-Collagen (Murray et al., 1995, 1:10), mouse monoclonal anti-Notch (Fehon et al., 1991, 1:50), and mouse monoclonal anti-NUBBN (Averof and Cohen, 1997, 1:20). After the incubation in primary antibody, legs were washed and blocked again as above, and then incubated in secondary antibody overnight (1:100 fluorescently labeled goat-anti-rabbit or mouse secondary antibodies from Jackson Laboratories) at 4°C or RT.

To visualize leg cell nuclei, propidium iodide was added to a final concentration of 0.01 mg/ml and deoxyribonuclease (DNase)-free ribonuclease (RNase) to a final concentration of 0.1 mg/ml to

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the secondary antibody solution. To see cell outlines, legs were washed several times with PBS after the secondary antibody incubation and incubated in the membrane dye FM4–64 (Molecular Probes; 0.16 mM in PBS) overnight at 4°C or RT. To stain filamentous actin, Oregon Green phalloidin (Molecular Probes) was used similarly at a final concentration of 0.016 U/mL in PBT. After the secondary antibody/cell marker incubation, the tissue was washed twice more in PBT and then mounted in Vectashield Mounting Medium for Fluorescence (Vector Laboratories). Images were collected with a Leica SP confocal microscope controlled by Leica TCS NT software.

Enhancer Trapping

We conducted a GAL4 enhancer trap screen, similar to the screen conducted by Brand and Perrimon (1993), to identify inserts showing expression in the pupal joints. To generate new GAL4 inserts, virgin females from the GAL4 line MSl096 (a gift from Dr. A. Brand), which carries a w; P{GawB} insert on the X chromosome, were crossed to males from a stock that carried a source of transposase (yw; 32,3 Stubble/TM6B) (Brand and Perrimon, 1993). F1 white Stubbles were collected and crossed to virgin females from a multiply balanced stock (yw; Sco/Cyo; TM2/TM6B). In about 1% of F2 males, P{GawB} transposed from the X onto another chromosome, these males had red eyes. A total of 312 red-eyed males were collected. These were crossed individually to w; Sco/Cyo; TM2/TM6B virgin females to determine which autosome carried the insert. F2 male progeny resulting from this cross were mated to w--;UAS-GFP virgin females (a gift from Dr. A. Brand). Pupae from this cross were collected every 24 h, allowed to develop until they were between 24 and 48 h AP, then dissected and fixed in 4% paraformaldehyde in PBS. The GFP expression pattern was assessed under epifluorescence. We also examined 194 GAL4 lines generated in Dr. O’Kane’s laboratory in a similar manner. Six lines showing expression in pupal joints were selected for further study.

Plasmid Rescue

The P{GawB} plasmid and flanking genomic sequences were rescued from each enhancer trap line essentially as described by Wilson et al. (1989). Plasmid and flanking DNA was sequenced by using a primer to the 3’ end of the P{GawB} construct (AAT TAA CCC TCA CTA AAG GG, the T3 promoter sequence from Bluescript). The site of each P{GawB} insertion was identified by conducting a BLAST search (Altschul et al., 1990) against the Drosophila Genome Database.

RESULTS

Natural History of Joint Formation

Drosophila legs have 10 joints that separate the leg segments from the body wall and from each other (Fig. 1a). This study focuses on the distal leg segments, especially the tibial/tarsal joint, but also the joint between the first and second tarsal segments (tar1/tar2 joint). We chose these joints because they are easily accessible both in the adult and the pupa. Although the joints along the leg vary in their specific morphology, we believe that events in these joints are representative of what happens in the remaining leg joints.

Images from the SEM capture the intricate structure of the adult joints. The tibial/tarsal joint is almost bilaterally symmetrical with the anterior and posterior sides being more similar to one another than they are to the dorsal and ventral sides (Fig. 1). On both anterior and posterior sides, the hard parts of the tibia extend distally to make contact with the opposing parts of the first tarsal segment (tar1) (Figs. 1b and 1c). On both anterior and posterior sides of tar1, there is a small ridge where the tibial extension fits on top of the proximal tarsus (Figs. 1b and 1c, arrow).

Tibia and tar1 do not come into contact on the dorsal side of the joint. Instead, the hard edge of the tibia arches away from the tarsus, revealing the folds of intersegmental membrane (Fig. 1d). These folds tuck in underneath the tibia, forming the internal structures of the joint. On the tarsal end of the joint, the leg shaft continues tube-like right up to the intersegmental membrane.

The morphology of the ventral side of this joint varies for the different legs (Figs. 1e and 1f). In the first and second legs, the ventral side of the tibial/tarsal joint resembles the dorsal side, except that the tibia curves away from the tar1 segment less steeply. In the third leg, the ventral side has an additional pair of stout projections that probably contact one another when the joint flexes ventrally (asterisk in Fig. 1f). The function of these structures may be to prevent the joint from bending too far in this direction.

Unlike the tibial/tarsal joint, the exterior surface of the tarsal joints is almost radially symmetrical (Figs. 1e–1g). The presence of campaniform sensillae on the dorsal side of the joint is one of the few features that distinguishes the different sides. The joint circles the leg on a slight angle with the dorsal side tilting more proximally than the ventral (Figs. 1e–1g). The inflexible portions of the proximal and distal ends are loosely apposed around the entire circumference, but the proximal segments do not extend and do not fit into ridges on the distal segments. The intersegmental membrane cannot be seen; it is tucked inside the shafts of the leg.

Joint Development

After the pupal cuticle is shed, at 18 h AP, the leg is little more than a long bloated sack. Between 18 and 20 h AP, the leg shrinks down to its final diameter and constricts further in the regions where the joints will form (Fig. 2) (Fristrom and Fristrom, 1993). A blunt protrusion at the end of the leg marks the developing pretarsus. By 24–26 h AP, constrictions in the joints are more prominent (Fig. 2). The hooks of the claw (ungui) are beginning to protrude from the end of the leg (arrow in Fig. 2d). From 27 to 29 h AP onwards, it is apparent that different joints are forming at different rates (Fig. 2). We first discuss the development of the tibial/tarsal joint and then highlight the similarities and differences between this joint and the tarsal joints during their development. We have not examined in detail the development of the other joints.
FIG. 1. SEMs of the structure of the adult tibial/tarsal and tar1/tar2 tarsal joints. (a) Whole leg showing all leg segments. The two white boxes mark the tibial/tarsal joint (box on the top) and the tar1/tar2 joint. Anterior (b) and posterior (c) views of the tibial/tarsal joint showing the points of contact between the proximal and distal joint surfaces (arrow in b and c). The joint rotates on these points of contact. On the dorsal (d) and ventral (e and f) sides of the joint, proximal and distal surfaces do not articulate. The flexible intersegmental membrane is visible in the dorsal view. Tibial/tarsal joints on the metathoracic leg have additional projections on the ventral side (asterisk in f). The tar1/tar2 joint (g-i) is much simpler. There are two campaniform sensillae (arrowheads in g-i) on the dorsal side of the joint. Otherwise, all sides look similar.
The Tibial/Tarsal Joint

After the initial constriction in the regions of the joints, tissue begins to fold into the leg at different rates around the circumference of the joint. At 27–29 h AP, lips on the lateral sides of the tibial/tarsal joint form where distal joint cells are folding in under the proximal cells, while the dorsal and ventral surfaces remain smooth (Fig. 2). At the same time, bristles begin to differentiate (Fig. 2). As the joint continues to differentiate over the next 3 h, more dorsal and ventral tissues fold inwards until all the distal joint tissue around the circumference of the joint has tucked into the tube of the leg. At 32–34 h AP, tibial/tarsal joints are nearly fully formed. Morphogenesis is complete by 36–38 h AP (Fig. 2).

We characterised cell shape changes during joint development using flies carrying the enlacZ reporter and a membrane dye, FM4-64. In the tibial/tarsal joint, apodemes extend from both the dorsal and ventral surfaces of the joint. Apodemes are tubes of cells that invaginate into the leg, forming projections of epidermis to which the muscles attach. The origins of these apodemes form convenient markers to define fixed points during subsequent joint morphogenesis. If the leg is oriented with the dorsal and ventral surfaces facing to the right and left, the apodemes can be seen as small openings in the epidermal surface, one each on the dorsal and ventral sides of the joint, that lead to a bilayered strip of cells running into the leg (see double arrowheads in Fig. 6b). All the cells level with and distal to the bottom surface of the apodeme we term distal joint cells; the cells above and including the proximalmost surface of the apodeme we term proximal joint cells. As we will see, this division into proximal and distal joint based on the location of the apodemes corresponds with populations of cells that both behave differently and express different joint markers.

We distinguish three stages of joint development, each

FIG. 2. Nomarski images of developing tibial/tarsal and tarsal joints. (a, c, e, g, i, k) Tibial/tarsal joints. (b, d, f, h, j, l) Tarsal joints. Proximal is towards the top of the page. (a, b) 18–20 h after pupariation (AP). Both sets of joints are only visible as slight indentations in the leg. The hooks of the claw are already growing out of the end of the leg (arrow in b). (c, d) 24–26 h AP. Constrictions where the joints will form become more prominent. The hooks of the claw continue to extend (arrow in d). (e, f) 27–29 h. In the lateral parts of the tibial/tarsal joint, distal cells begin to fold in (e), while the tarsal joints remain unchanged (f). Bristles are beginning to protrude from the tarsi (arrowhead in f). (g, h) 30–32 h AP. Invaginations in the tibial/tarsal joint extend around the circumference of the leg (g). The tarsal joints have not begun to fold under (h). The pulvilli of the claw are apparent as beaded protrusions from the end of the leg (asterisk in h). (i, j) 32–34 h AP. The tibial/tarsal joint is almost complete (i), but although more bristles are visible in the tarsi, these joints have still not progressed (j). (k, l) 36–38 h AP. Morphogenesis of all the joints, the bristles and the claw organ is complete. Scale bars, 15 μm.
FIG. 3. Cell shape changes in the differentiating tibial/tarsal joint. Legs from flies carrying enlacZ, which marks the posterior compartment, were stained using anti-β-gal (green) and a cell outline marker (FM 4-64). (a–d) Legs at 18–20 h AP. Joint morphogenesis has not yet begun. (e–h) Joints at 24–26 h AP. Cells below the apodeme (arrowhead in e), i.e., distal joint cells (enclosed by brackets in e and f), constrict apically (previously described by Fristrom and Fristrom, 1993). f' ) and f'' ) are magnification of joint and nonjoint cells from (f), respectively. (i–l) At 27–29 h AP, the distal joint cells become columnar and adhere closely together. (m–p) Joint morphogenesis is complete at 36–38 h. Cells in the proximal joint align and form a “palisade,” which stretches over the distal joint cells. Arrowhead in (o) marks the proximal end of an apodeme originating in the tarsi. The scale bars are 20 μm.
with its own populations of cells behaving in a reproducible manner. Here, we present four time points for each joint to represent these three stages of joint morphogenesis; a more detailed time series for both the tibial/tarsal and tar1/tar2 joint development is available on request.

During the first stage, at 24 h AP, the apical surfaces of joint cells constrict (Fristrom and Fristrom, 1993). This occurs in all joints, simultaneously producing indentations along the length of the leg. In the tibial/tarsal joint, cells below the apodeme insertion site (cells between the brackets in Figs. 3e and 3f) appear smaller in circumference than the opposing cells in surface sections (compare joint cells in Fig. 3f to nonjoint cells in Fig. 3e). This first stage is most evident in SEMs (see Fig. 4).

In the second stage, beginning at 27–29 h AP, cells in the lateral anterior and posterior domains in the distal part of the tibial/tarsal joint become more columnar and pack tightly together in comparison to the nonjoint cells (Figs. 3k and 3l). Presumably, apical constriction and elongation of distal joint cells cause the contractions in the joints seen at these stages under the light microscope. As joint morphogenesis progresses, the folding of distal joint tissue into the leg produces a C-shaped curve with elongated cells around all sides of the invagination (Figs. 3k and 3l and 3o and 3p; 27–29 h AP). This cup of tissue deepens and becomes directed proximally as joint differentiation proceeds.

Finally, between 32 and 34 h AP and 36 and 38 h AP, cells on the proximal edge of the fold align and flatten in a proximal–distal direction, forming a “palisade” (a row of columnar cells) over the distal joint cells (stage 3, Figs. 3o and 3p). This alignment of cells at the proximal edge of the fold is reminiscent of the leading edge cells during dorsal closure (Young et al., 1993; Martinez Arias, 1993).

The cell shape changes documented here account for the general morphology of the tibial/tarsal joint, but there must be events occurring after 36–38 h AP that generate additional features of the adult joint. For instance, at 36–38 h AP, we are still not able to discern the folds of intersegmental membrane. We have not investigated these later stages; from 38 h AP onwards, the secreted cuticle prevents the use of membrane dyes and confocal microscopy.

**Tarsal Joint Development**

Tarsal joints begin morphogenesis at the same time as the tibial/tarsal joint (24–26 h AP; Fig. 2), but they progress more slowly and then rapidly complete invagination at 36–38 h AP (Fig. 2). The only exception is the joint between the fifth tarsal segment and the pretarsus, which completes its differentiation by 34–36 h AP (data not shown).

The tarsal joints do not have apodemes, but we were able to correlate the cell shape changes we saw in the tibial/tarsal joint with those in the tarsal joints. We therefore assume that these cell behaviours define the corresponding domains in the tarsal joints.

Similar cell shape changes occur in both the tibial/tarsal and the tar1/tar2 joint, but stages 2 and 3 occur later in the tarsi. Distal tar1/tar2 joint cells constrict apically at 24–26 h AP (stage 1, Figs. 5e and 5f) (Fristrom and Fristrom, 1993). However, the distal cells in the lateral anterior/posterior domains do not appear tightly packed until 30–32 h AP (stage 2, Figs. 5e and 5f). This joint undergoes no further morphogenetic movements until shortly before 36–38 h AP, when the distal joint cells fold in and the cells on the proximal end of the joint align at the edge of the fold (stage 3, Figs. 5m and 5n).

The morphogenesis of other distal structures also occurs between 24 and 36 h AP. In the pretarsi, the claws (or ungui) begin to extend at 24–26 h AP and reach their final length and shape at 36–38 h AP (Figs. 2d, 2f, 2h, 2j, and 2l). Other parts of the pretarsi, such as the knobby projections of the developing pulvilli and empodium, appear at 30–32 h AP (Fig. 2h).

**The Cell Machinery Is Modified during Joint Formation**

To assess the organisation of the cytoskeleton and the extracellular environment of joint tissue, we investigated microfilament and extracellular matrix distribution in legs undergoing joint morphogenesis. Legs stained both with Oregon Green phalloidin, marking the location of filamentous actin, and the cell membrane marker FM4-64 show that the actin cytoskeleton is differentially organised in joint and shaft cells during joint differentiation. The distribution of collagen IV changes as the joints develop, but that of laminin does not.

At 18 h AP, when the leg is constricting throughout its length to reduce its diameter (Fristrom and Fristrom, 1993), filamentous actin is present at high levels in all leg cells.
Nubbin (Nub). Of all the markers examined, only Nubbin or the distal territories (e.g., Notch and odd-skipped lacZ) are expressed in more than two joints in the L3 stage. Others mark one or two joints at this stage but are expressed in all joints during the pupal stage (see Table 1 for details). Previous studies examining the expression of Notch and other elements of the Notch patterning cascade have also found that the joint seems to be divided into proximal and distal territories at this stage (de Celis et al., 1998, Rauskolb and Irvine, 1999). Thus, proximal and distal joint domains have already been established by the late L3.

By 34–38 h AP, patterns of marker expression define three additional territories. First, a proximal–dorsal patch was highlighted by two joint markers, ckm90 and ckm175, that drive GFP expression only in a patch above and including the most proximal cells of the dorsal apodeme. The expression of GFP driven by ckm175 includes a greater number of cells than that driven by ckm90 (Fig. 8, and Table 1). The second domain identified was a mid-distal domain. Odd lacZ expression becomes largely restricted to a mid-distal group of cells in all but the tarsal joints. This corresponds to the region that does not accumulate collagen IV and marks the cells that push underneath the proximal joint cells. Odd lacZ is also expressed in the apodemes. Lastly, ok388 expresses GFP in the lateral anterior and posterior parts of the distal tibial/tarsal (but not tarsal) joint, but is excluded from the dorsal and ventral domains (Fig. 8, and Table 1). This expression domain corresponds with the region of elongating cells seen in longitudinal sections of the leg (see Fig. 9).

Two of the joint markers are expressed in both the proximal and distal portions in the developing adult joint: ckm239 and disco lacZ (Fig. 8, and Table 1). disco lacZ is expressed throughout the entire joint, and ckm239 is excluded from the ventralmost region (wingless lacZ-expressing region).

**Joint Territories**

To identify distinct cell populations in the joints, we examined the expression patterns of 10 joint markers with respect to a posterior marker (engrailed lacZ) and a ventral marker (wingless lacZ). We examined the leg discs of wandering larvae, and pupal legs at 24–28 and 34–38 h AP. Four of the joint markers were previously reported to be expressed in L3 and prepupal joints (Notch, disconnected lacZ, Nubbin, and odd-skipped lacZ). The rest were isolated for this study by screening Gal4 enhancer trap lines for those that drive expression of GFP in pupal leg joints (ckm78, ckm90, ckm239, ckm175, ok388, and ok483; see Materials and Methods). Most of the joint markers do not change their expression domains between 24–28 and 34–38 h AP. Therefore, we only present data from wandering L3 discs, and from legs at 34–38 h AP.

In the L3 leg disc, joint markers fell into one of two categories, marking either the proximal joint territories (e.g., Nubbin) or the distal territories (e.g., Notch and odd-skipped lacZ). Of all the markers examined, only Nubbin (Nub), disconnected lacZ (disco lacZ), and odd-skipped lacZ (odd lacZ) are expressed in more than two joints in the L3 stage. Others mark one or two joints at this stage but are expressed in all joints during the pupal stage (see Table 1 for details). Previous studies examining the expression of Notch and other elements of the Notch patterning cascade have also found that the joint seems to be divided into proximal and distal territories at this stage (de Celis et al., 1998, Rauskolb and Irvine, 1999). Thus, proximal and distal joint domains have already been established by the late L3.

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**Plasmid Rescue of Joint GAL4 Lines**

For each GAL4 line, we obtained both the cytological location of the inserted P element and the precise location of its 3′ end in relation to the genome sequence (Table 2). Of the six joint markers isolated from the enhancer trap screen, four are inserted in the vicinity of a gene previously described in the literature (ckm90, ckm175, the sequence from ckm239 in the 72D1 region, and ok483; Table 2). However, none of these genes provides an obvious candidate for involvement in joint morphogenesis. The remaining three lines, ckm90, ok388, and ok483, are inserted more than 17 kb away from any identified coding region.

**DISCUSSION**

**Joint Morphogenesis: Correlating Expression Domains and Cell Behaviour**

The differentiating joint is characterised by three distinct phases of cell behaviour (Fig. 10).
FIG. 5. Cell shape changes in the differentiating tar1/tar2 tarsal joint. Legs from flies carrying enlacZ, which marks the posterior compartment, were stained using anti-β-gal (green) and a cell outline marker (FM 4-64). (a–d) Legs at 18–20 h AP. Joint morphogenesis has not yet begun. (e–h) Joints at 24–26 h AP. Distal joint cells (enclosed in brackets in e and f) constrict apically (previously described by Fristrom and Fristrom, 1993). (i–l) 30–32 h AP. Distal joint cells become columnar and adhere closely together. (m–p) 36–38 h AP. Joint morphogenesis is complete. Cells in the proximal joint have aligned and formed a "palisade," which stretches over the distal joint cells. The scale bars are 20 μm.
Stage 1: Distal joint cells constrict their apical surfaces, causing an indentation in the tube of the leg where the joint will form.

FIG. 6. Changes in filamentous actin distribution during tibial/tarsal joint morphogenesis. All legs are from wild-type flies stained with FM4-64 (membrane dye, red) and Oregon Green phalloidin. For all images, the proximal part of the leg is towards the top of the page. The double arrowheads in (b), (c), (e), and (h) mark the insertion sites of apodemes (which insert on the dorsal and ventral sides of the legs). The short arrows in (b), (d), (f), and (h) show the basal cell surface of one side of the joint epithelium. (a, b) Legs at 18 h AP. Filamentous actin is present at high concentrations throughout the leg and is especially high in the apodemes. Cells accumulate filamentous actin in apical, basal, and lateral membranes (b' is a magnification of cells from b). (c, d) 24 h AP. Filamentous actin is present on the apical and basal but not lateral surface of cell membranes (d' is a magnification of cells in d, compare b' with d'). (e, f) 30 h AP. Filamentous actin accumulates in the cells that are folding inwards on the anterior and posterior sides of the joint (long arrow in e and f). (g, h) 36 h AP. Filamentous actin continues to accumulate in the invaginating cells (long arrow in g and h). Actin levels are also high in the elongated cells in the proximal joint. Scale bars in (a–d) are 50 μm, in (e) and (f) are 10 μm, and in (g) and (h) are 20 μm.

FIG. 7. The distribution of the ECM proteins laminin and collagen IV in the tibial/tarsal joint during morphogenesis. (a, b) Red is propidium iodide (marks nuclei) and green is an antibody stain against laminin. (c, d) Red is FM4-64 (marks cell outlines) and green is an antibody stain against collagen IV. For all images, proximal is towards the top of the page. Laminin is strongly but evenly expressed on the basal membrane inside the leg both at 24 (a) and at 31–34 h AP (b). At 18 h AP, collagen IV is evenly but weakly distributed along basal cell surfaces in the leg (c). Twelve hours later (d), collagen IV accumulates at the basal surface of distal joint cells but is expressed less strongly in the extracellular matrix of other leg cells. Arrowheads in (d) mark where distal cells begin in the joint. Scale bars are 20 μm.
Stage 2: Distal cells on the anterior and posterior sides of the leg become columnar, and perhaps adhere more closely, causing the tissue to bend into the leg. Under the light microscope, this behaviour is apparent as a lip of tissue extending first over the distal joint cells in the lateral sides of the joint and then over all distal joint cells.

Stage 3: The first row of proximal cells on the edge of the fold extends in a proximal–distal direction and aligns as a “palisade.” Initially this happens uniformly around the circumference of the leg, but subsequently cells in the dorsalmost and ventralmost portions of this fold retract a little, thereby causing this tissue to arch, a feature of the adult dorsal and ventral joint structures (Figs. 6g and 6h).

These cell behaviours correlate with some of the marker expression domains observed at 34–38 h AP (Figs. 11d and
Notch and ckm78 mark the distal cells that constrict apically and become columnar. Within the distal joint, the mid-distal cells that fold underneath the proximal leg tissue are those marked by odd lacZ. These cells accumulate filamentous actin and have less collagen IV in their basal lamina. The distalmost joint cells narrow but do not fold into the leg. These cells also accumulate high concentrations of collagen IV in their basal lamina. In the lateral anterior and posterior sides of the distal joint, marked by ok388, cells become columnar and pack closely together. This distal-lateral tissue folds more deeply into the joint than the distal dorsoventral tissue. The proximal cells closest to the edge of the fold align, flatten, and accumulate filamentous actin during joint morphogenesis. They are within the Nub and ok483 expression domain, but we have not identified any markers exclusively in these aligning cells.

Not all markers define cell populations that correlate with identifiable cell behaviours. The dorsal patch of cells in the proximal joint, marked by ckm90 and ckm175, does not differ obviously in its morphology or behaviour from the surrounding cells. The expression of these markers may either reflect structures that are to differentiate later in pupal development or indicate more about how gene expression is regulated in the joint than about actual joint structure.

Most of the markers examined are expressed either in proximal or distal joint tissue, but two markers, disco lacZ and ckm239, encompass both regions. Why joint cells might require "general" joint identity is unclear, especially considering that different parts of the joint perform such different tasks. The expression of both of these markers arises after joint identity is determined; disco lacZ is not expressed until after joint cells have been singled out, and

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression pattern in L3</th>
<th>Expression pattern at 4-6 h AP</th>
<th>Expression pattern at 34-38 h AP</th>
<th>Expressed in both tibia and tarsal joints?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disconnected LacZ</td>
<td>expressed in rings in the distal leg with fainter expression in proximal leg tissue</td>
<td>rings in joints*</td>
<td>expressed throughout the joint</td>
<td>yes</td>
</tr>
<tr>
<td>Nubbin</td>
<td>rings in the proximal joint</td>
<td>same as L3b</td>
<td>proximal joint tissue and subset of bristle lineage</td>
<td>not in tarsal joints</td>
</tr>
<tr>
<td>Odd-skipped LacZ</td>
<td>rings in the distal joint</td>
<td>same as L3b</td>
<td>mid-distal joint tissue and apodemes</td>
<td>not in tarsal joints</td>
</tr>
<tr>
<td>Notch</td>
<td>ubiquitously expressed</td>
<td>rings in distal joint tissuec</td>
<td>distal joint tissue and apodemes</td>
<td>yes</td>
</tr>
<tr>
<td>Ckm78</td>
<td>In proximal leg tissue and in one ring in the tarsi</td>
<td>same as L3</td>
<td>distal joint tissue, socket cells of the bristles, some expression in shaft</td>
<td>yes</td>
</tr>
<tr>
<td>Ckm90</td>
<td>no expression</td>
<td>no expression</td>
<td>proximal-dorsal patch in joint</td>
<td>yes</td>
</tr>
<tr>
<td>Ckm175</td>
<td>ubiquitous</td>
<td>rings in joints</td>
<td>proximal-dorsal patch in joint</td>
<td>yes</td>
</tr>
<tr>
<td>Ckm239</td>
<td>ubiquitous except within the wingless lacZ expressing region of the disc</td>
<td>complicated patchy pattern</td>
<td>expressed throughout the joint except in the wingless lacZ domain, expressed at lower levels in the shaft cells</td>
<td>yes</td>
</tr>
<tr>
<td>Ok388</td>
<td>throughout the anterior and posterior regions of the tarsi</td>
<td>same as L3</td>
<td>Distal-lateral joint, in bristles in tarsis</td>
<td>not in tarsal joints</td>
</tr>
<tr>
<td>Ok483</td>
<td>two tarsal rings</td>
<td>Rings in the T3 and T5/pretarsal region and a broad band in tibial and tar1</td>
<td>Proximal joint, in shaft region below joint and in shaft and bristles in tarsi</td>
<td>not in tarsal joints</td>
</tr>
</tbody>
</table>

* Bishop et al. (1999).
* de Celis et al. (1998).
ckm239 does not upregulate GFP in the joints until after 28 h AP. Perhaps these genes act in a manner similar to hairy in the prepupal discs, to suppress the development of bristles or other nonjoint structures in the joint (Orenic et al., 1993).

The Difference between Joints

The same stages of cell behaviour are observed in both tibial/tarsal and tarsal joints, and at least the Notch signalling pathway seems to be involved in the formation of all joints (de Celis et al., 1998, Rauskolb and Irvine, 1999, Bishop et al., 1999). However, several markers, such as odd lacZ, Nub, ok388, and ok483, are expressed in all the joints of the leg except the tarsal joints. Other markers not examined here, such as A101 lacZ and deadpan lacZ (Marion Rozowski, personal communication), are expressed only in the tarsal joints.

Some of these markers may define genes that specify the differences between adult tibial/tarsal and tarsal joint structures. For example, the marker ok388, expressed in the anteroposterior lateral region, is expressed in tibial/tarsal but not the tarsal joints, possibly because the tibial/tarsal joints have distinct lateral (anterior/posterior) and dorso-ventral domains. The tarsal joints are more radially symmetric.

However, the differences between tibial and tarsal joints may have less to do with joint structure than with the evolutionary history of these joints. Comparison of leg structure between insects and other arthropod groups suggests that the tarsal joints were acquired independently of the more proximal joints, and more recently (Snodgrass, 1935). The expression of Notch pathway genes in both types of joint suggests that they share at least this component of the patterning mechanism, but other components of the mechanism may have been recruited independently.

Joint Polarity and the Proximodistal Axis

The different behaviours of proximal and distal cells in the joint illustrate that proximodistal orientation within the joint is critical to the differentiation of a normal joint. The proximodistal orientation of the joint is likely to arise from the same mechanisms that provide proximodistal orientation to the whole leg. These early patterning events position the joints and, probably, set up planar polarity signals within the epidermis. It is not clear whether joint polarity is established directly by the specific sequence of abutting proximodistal leg territories, or by an indirect influence of epithelial planar polarity on the joint. For the tarsal joints, the latter seems to be more likely. Several mutations that cause planar polarity reversals in the epidermis also cause reversals in joints, so that the proximal joint tissue forms at the distal end of the joint (Held et al., 1986). Mutations in disheveled cause ectopic tarsal joints with reverse orientation. Furthermore, in these ectopic joints, Notch and Delta lacZ are expressed in the opposite order, with Notch lacZ expressed more proximally (Bishop et al., 1999).

When Are Joint Territories Defined?

It seems likely that the domains of gene expression observed in the L3 leg disc correspond with those of the same genes in the developing adult joint, though we have not verified this directly. If so, proximal and distal joint domains are established before pupariation. These two joint territories separate cells that will invaginate [the cells in the odd lacZ domain, expressing the Notch target E(SPL)Mβ] from those that will form the proximal palisade (the cells expressing Delta, Serrate, and Numbin) (Fig. 11).

During pupal development, the proximal and distal domains of the joint become further subdivided (Figs. 11b and 11c). Most of the enhancer trap markers that we have...
Pink= markers in the proximal joint (ok 483 and Nubbin)
Yellow= markers in a patch of proximal-dorsal joint cells (ckm90 and ckm175)
Blue= markers in the distal joint (Notch and ckm78)
Light Blue= ckm239, upregulated in the joint everywhere except the ventral stripe
Purple= markers in the mid-distal joint (odd-skipped lacZ)
Green= markers in the distal joint cells on the anterior and posterior lateral sides (ok388)

P= posterior
D= dorsal
A= anterior
V= ventral
FIG. 10. Cell shape changes during joint morphogenesis. Diagrams of surface sections (a, c, e) and longitudinal sections (b, d, f) at 24–26 (Stage I), 27–29 (Stage II), and 36–38 h AP (Stage III) in the tibial/tarsal joint. Proximal is towards the top of the page and dorsal is projecting out of the page. The yellow dot is the insertion point of the dorsal apodeme. Stage I: Distal joint cells are smaller in surface section. Stage II: Elongated, columnar cells are visible in the distal anterior and posterior regions. Stage III: Joint morphogenesis is complete. Proximal cells along the edge of the fold have elongated and aligned in surface section. Distal cells are constricted, and the distal anterior and posterior cells around the invagination are elongated. Similar shape changes were observed in the tarsal joints.

FIG. 11. Joint territories in relation to cell shape changes during morphogenesis. (a) L3 territories projected onto the early pupal joint. (b) Territories at 24–26 h AP in the tibial/tarsal joint. (c) Territories at 36–38 h AP in the tibial/tarsal joint. (d, e) Cell outlines are plotted diagrammatically onto surface sections (d) and longitudinal sections (e) of 36- to 38-h joints. In all images, the yellow dot represents the insertion point of the dorsal apodeme; in (d), this apodeme inserts between the palisade cells and the distal joint tissue that has folded in behind the proximal joint cells. Dashed lines in (e) represent the boundaries of the different joint territories. (a–c) Dorsal views. Color code: pink, proximal joint cells (express GFP in ok483 and Nubbin); blue, distal joint cells (express Notch and GFP in ckm78); dark blue, mid-distal joint cells that fold in under the proximal joint cells (marked by odd lacZ expression); green, distal anterior and posterior cells that elongate and express GFP in ok388. None of the markers characterised in this work are expressed exclusively in the proximal cells that align along the fold.

FIG. 9. Expression patterns of joint markers at 34–38 h AP. In all images, green indicates the joint marker [anti-β-gal in a, anti-PDM in e, anti-NOTCH in b, or green fluorescent protein (GFP) in c, d and f–j]. In (e), red is anti-β-gal staining in an odd lacZ line. In all other images, red is propidium iodide. In (c, d) and (f–j), blue is anti-β-gal in a wingless lacZ line. Diagram (k) represents all of the different cell populations in the joint revealed by these joint markers. The scale bar is 20μm.
identified are expressed in specific groups of cells within either the proximal or distal domain in the tibial/tarsal joint at 34–38 h AP. At the same time, the expression of some earlier markers becomes restricted to more specific territories. odd lacZ, which is expressed in some joints in the L3, is expressed most strongly in the mid-distal joint cells at 34–38 h AP. Ok388 expresses in the distalmost but not mid-distal joint cells, and is restricted to the lateral anterior and posterior sides. In the proximal joint, markers such as ckm90 and ckm175 express in only a small group of cells on the dorsal side. Thus, it seems that the tibial/tarsal joint may divided into three proximodistal domains based both on cell behaviour and gene expression: proximal, mid-distal, and distalmost regions. Later during pupal development, the distalmost region subdivides into lateral anterior/posterior and dorsal/ventral domains and the proximal joint also subdivides into smaller territories (Fig. 11). That further patterning and subdivision of the joint occurs after the prepupal stages is hardly surprising: the adult joint is too complex a structure to be derived simply from the proximodistal interactions that occur before pupal development.

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