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The Role of Teashirt in Proximal Leg Development in *Drosophila:* Ectopic *teashirt* Expression Reveals Different Cell Behaviours in Ventral and Dorsal Domains

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Localised transcription factors specify the identity of developmental domains. Here we analyse the function of the Teashirt zinc finger protein, which is expressed in the proximal domain of the *Drosophila* leg. By ectopic expression of a *teashirt* transgene we show that Teashirt contributes to the differences in cell–cell adhesion between proximal and distal leg cells. Whereas clones of cells expressing the *teashirt* transgene survive in the endogenous Teashirt domain, most cells expressing Teashirt in an ectopic distal position are lost from the epithelium. In clones which were recovered in the distal domain, different effects were seen dependent on position with respect to the dorsal–ventral axis. In the ventral region, where Wingless is signalling, surviving clones express Teashirt and cause abnormalities in the adult leg. Contrarily, lateral and dorsal clones generally do not accumulate Teashirt and have no effect on patterning. One exception to the differential dorsal–ventral effects occurs at the boundary between Teashirt-expressing and -nonexpressing cells. Both ectopic and hypomorphic loss of *teashirt* affects patterning at all positions at the boundary, suggesting that Teashirt plays a crucial role in boundary formation. The results are discussed with respect to the roles of transcriptional and posttranscriptional mechanisms in proximal–distal axis patterning of the *Drosophila* legs.

Key Words: **Teashirt zinc finger protein; proximal–distal leg boundary; differential cell mixing; protein accumulation; cell interactions.**

INTRODUCTION

The *Drosophila* leg derives from a small group of cells in each hemisegment of the embryonic thorax (Cohen, 1993). These cells divide during the larval stages to form the leg imaginal discs, consisting of a single layer of epithelial cells forming a sack (Figs. 1A and 1B). During pupal development the discs evaginate to form the adult legs (Fig. 1C). Each leg disc gives rise to the body wall, coxa, and trochanter (the proximal parts) and the femur, tibia, and tarsus (distal parts). These domains develop from distinct rings of cells mapped onto a fate map of the mature discs (Schubiger, 1968). Proximal structures derive from the periphery and peripodial membrane of the disc and the most distal structures from the central cells (Fig. 1).

Distinct domains of cells may develop in a lineage-dependent way, such as the anterior and posterior compartments of the *Drosophila* wing (Garcia-Bellido, 1975), or in a lineage-independent manner, for example dividing the *Drosophila* leg in proximal and distal domains (Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999).

Different domains are separated by boundaries. Boundary formation is well understood for the establishment of the anterior–posterior (A-P) axis of the limbs (Blair and Ralston, 1997; Garcia-Bellido, 1975; Rodriguez and Basler, 1997), whose maintenance relies on transcriptional regulation and long- and short-range cell signalling as well as differential cell mixing properties between anterior and posterior compartments. Lineage-independent boundaries separating the

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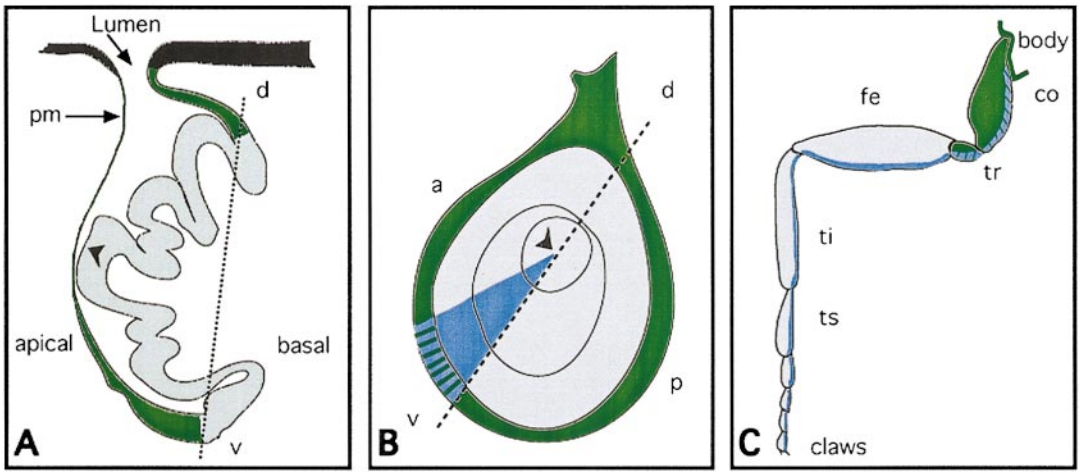

FIG. 1. Schematic representation of *Drosophila* leg discs and the adult leg (Schubiger, 1968; modified from Fristrom and Fristrom, 1993). (A) A dorsal–ventral (d-v) cross section through a third-instar leg imaginal disc, which is made up of a folded epithelium inside the larva, attached to larval structures (black shading). The peripodial membrane (pm) overlays the distal epithelium (arrowhead) and corresponds to the most proximal structure of the disc, which will form parts of the future body wall. The green-shaded regions of the epithelium will give rise to the body wall, coxa, and trochanter. The dotted line represents the focus on the P-D boundary (between trochanter and femur). Note that in our experiments (Figs. 2, 4, 5, and 6), discs are mounted and become flattened compared to this diagram. (B) Third-instar imaginal disc with an apical view (under the peripodial membrane). The arrowhead indicates the distal tip. The proximal regions (green) derive from the ring on the periphery at the disc. The dotted line corresponds to the anterior (a)–posterior (p) compartment boundary. The blue region represents the expression domain of *wg*, in the ventral anterior sector. Wg is secreted on either side of its expression domain to organize ventral and P-D patterning. (C) A *Drosophila* adult leg representing the different leg segments along the P-D axis: the proximal segments (green) are the coxa (co) and the trochanter (tr). The distal segments are called the femur (fe), tibia (ti), and tarsus (ts). The ventral region is shown by the blue shading. The leg is attached by the coxa to the body wall.

proximal–distal domains of the *Drosophila* leg require additional unknown mechanisms involving cell–cell interactions (Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999).

Along the proximal–distal (P-D) axis of the developing *Drosophila* leg, different transcription factors are expressed in distinct and often partially overlapping domains (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). Homothorax (Hth), a homeodomain protein (Rieckhof *et al.*, 1997), and Teashirt (Tsh), a zinc finger protein (Fasano *et al.*, 1991; Gonzalez-Crespo and Morata, 1996), are expressed in the proximal domain of the developing leg. Dachshund (Dac), a nuclear protein (Mardon *et al.*, 1994), is expressed in an intermediate ring of cells (Lecuit and Cohen, 1997). Finally Distal-less (Dll), a homeodomain protein (Cohen *et al.*, 1989), defines the distal region of the leg. Dll has also a later expression domain, the proximal ring, required for the development of the trochanter and proximal femur (Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999).

Signalling pathways are crucial for the creation and/or maintenance of P-D domains. Wingless (Wg) and Decapentaplegic (Dpp) pathways initiate the leg primordia by switching on Dll (Goto and Hayashi, 1997). Later, Wg (Fig. 1B) and Dpp signalling are expressed in, and are necessary respectively to establish, the ventral and dorsal parts of the leg primordia (Brook and Cohen, 1996; Jiang and Struhl, 1996). The concentration of these morphogens (Neumann and Cohen, 1997) appears to be essential for organising the expression of specific transcription factors in distinct domains along the P-D axis. For example, the combination of low Wg and Dpp signalling in the same cells induces the expression of *dac*. Higher concentrations of these same morphogens induce the expression of *Dll* and the repression of *dac* (Lecuit and Cohen, 1997).

Each of the secreted proteins activates specifically a conserved signalling pathway (reviewed in Lawrence and Struhl, 1996; Wodarz and Nusse, 1998). In cells receiving the Wg signal, for example, Armadillo (Arm) accumulates inside cells, where it is then free to bind to *Drosophila* T cell factor (dTCF). Arm binds also to the intracellular domain of DE-cadherin, a transmembrane protein, and has an essential role for cell adhesion in all cells, irrespective of whether they receive Wg or not (Peifer and Wieschaus, 1990, Oda *et al.*, 1994; Cox *et al.*, 1996; Orsulic and Peifer, 1996).

Differential cell mixing is a crucial mechanism that keeps cells of different domains apart. This separation phenomenon relies on the activity of the locally expressed transcription factors, including Tsh (this work), Hth, and Dll (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999), in the developing *Drosophila* leg. Recently we have shown that Tsh binds directly to Arm *in vitro* and probably *in vivo* (Gallet *et al.*, 1998, 1999). This property may provide a way

of modulating both Wg signalling to the nucleus and the particular mixing properties of cells of the proximal leg domain.

By uncoupling the transcriptional from the posttranslational control of *tsh*, we show that Tsh-expressing cells have differential mixing properties compared to their distal neighbours. More importantly our results indicate the existence of two novel mechanisms required for the maintenance of a boundary between Tsh and Dll expression domains, one of which relies on Wg signalling.

MATERIALS AND METHODS

tsh Mutations

$tsh^{12\text{-}5}$ and tsh^{3} were induced by EMS and jump-start mutagenesis, respectively. *Df(2L)305* was a gift from Barbara Wakimoto and deletes *tsh* and other neighbouring genes. The three mutations were balanced with a $CyOy^{+}$ chromosome. UASTsh is described in Gallet *et al.* (1998). A *dacLacZ* transgene (dac^{P}; Mardon *et al.*, 1994) was used in wild type and in combination with $tsh^{12\text{-}5}/Df(2L)tsh^{305}$.

Dll⁻ Clones

Female $y^{1}\ w^{1118}\ P\{ry^{+t7.2} = hsFLP\}1/y^{1}\ w^{1118}\ P\{ry^{+t7.2} = hsFLP\}1;\ P\{ry^{+},\ hs\text{-}neo,\ FRT\}42D\ Dll^{SA1}/CyO$ were crossed to male $P\{ry^{+},\ hs\text{-}neo,\ FRT\}42D\ P\{arm\text{-}LacZ\ w^{+}\}/P\{ry^{+},\ hs\text{-}neo,\ FRT\}42D\ P\{arm\text{-}LacZ\ w^{+}\}$. Their progeny were heat shocked at 36°C at 72–120 h after egg laying to induce clones.

Flp-out Clones

Ectopic Tsh clones were obtained by using the "*flp*-out" system (Struhl and Basler, 1993). Clones were induced in larvae carrying three different transgenes. The first carries a *hsp70* promoter driving the yeast *flp* recombinase which mediates site-specific recombination between two *cis*-acting FRT (*flp* recombination target) sequences. The second transgene consists of a ubiquitous promoter placed upstream of the coding sequence of Gal 4 but separated from it by a "*flp*-out cassette." The *flp*-out cassette consists of a cellular marker, such as $yellow^{+}$ or $CD2^{+}$, cloned between two FRT sequences. The third transgene carries the binding sites of Gal4 upstream of a full-length *tsh* cDNA (UAS-Tsh; Gallet *et al.*, 1998). Following a heat shock at 36°C in these larvae, the *flp* recombinase is produced, removes the *flp*-out cassette, and leads to production of Gal4 and so to ectopic *tsh* transcription in single cells. This cell is recognised by loss of the cellular marker. After cell division clones of cells, ectopically producing Tsh can be observed. In our experiments we induced clones by hsFLP (located on chromosomes 1 or 2) using the *flp*-out Gal4 lines: $arm = y^{+} = VP16Gal4$ (M. Haenlin) on chromosome 3 or an $actin5C = CD2^{+} = Gal4$ (Pignoni and Zipursky, 1997) on the X chromosome. Ectopic UASTsh clones (with or without UASLacZ) were made by crossing $y\ w\ P\{ry^{+t7.2} = hsFLP\}22/y\ w\ P\{ry^{+t7.2} = hsFLP\}22;\ arm = y^{+} = Gal4VP16$ (or $y\ w\ actin = CD2^{+} = Gal4;\ P\{ry^{+t7.2} = hsFLP\}38/P\{ry^{+t7.2} = hsFLP\}38$) females to *UASLacZ/UASLacZ; UASTsh/UASTsh* males. Clones were induced at 48–72 or 72–96 h after egg laying at 36°C for 5 (short induction), 10 (medium induction), or 20 (longer induction) min. To produce double UASTsh and UASArmS10c (or UASWg) clones (short induction only) female $y\ w\ P\{ry^{+t7.2} = hsFLP\}22/y\ w\ P\{ry^{+t7.2} = hsFLP\}22;\ y\ w\ arm = y^{+} = VP16Gal4/arm = y^{+} = VP16Gal4$ were crossed to *UASWg/UASWg; UASTsh/UASTsh* (or *UASArmS10c/UASArmS10c; UASTsh/UASTsh*) males. Discs co-expressing ArmS10c and Tsh were stained with anti-Tsh and anti-Myc (a Myc tag is fused in frame to the COOH terminal end of the ArmS10c protein (Pai *et al.*, 1997)).

Immunostaining of Imaginal Discs

Mouse anti-Dll (from Stephen Cohen) was used at 1/1000. Mouse anti-βGal (Promega) was used at 1/500. Rat anti-Tsh was used at 1/600 (Gallet *et al.*, 1998). Mouse anti-Myc (9E10; Santa Cruz Biotechnology) was used at 1/100. Mouse anti-CD2 (Serotec) was used at 1/2500. Secondary FITC- or TRITC-coupled antibodies (Jackson Laboratories) were used at 1/100. Disc fixation and fluorescence labelling was performed as in Gallet *et al.* (1998) and Xu and Rubin (1993). A Zeiss confocal microscope was used for analysis.

In Situ Analysis and Histochemical Staining

tsh mRNA detection was accomplished as described in Fasano *et al.* (1991) and Gallet *et al.* (1998). X-Gal staining of leg discs from the dac^{P} line was performed as described in Fasano *et al.* (1991).

RESULTS

Teashirt and Distal-less Expression in the Developing Drosophila Leg

Legs derive from three pairs of primordia situated in the embryonic epidermis of the thorax. During the larval stages, these primordia divide to form imaginal discs (Figs. 1A and 1B) which give rise to the adult leg and body wall (Fig. 1C) after metamorphosis.

We have compared the expression domain of Tsh with that of Dll, a homeodomain-containing protein (Cohen *et al.*, 1989), at different developmental stages in the leg anlage (Fig. 2). At stage 10 of embryogenesis (Fig. 2A), Dll is detected in the putative distal part of the primordia of the leg in each of the thoracic hemisegments of the embryo (Goto and Hayashi, 1997). Tsh is coexpressed with Dll at this stage in a line of cells (Fig. 2A) that also produces the signalling protein Wg (Cohen, 1993). By stage 15 (Fig. 2B), the cells of the presumptive leg imaginal discs have invaginated inside the embryo and Tsh is not detected in the most distal part of the leg primordium, where Dll is expressed alone. However, Tsh is still coexpressed with Dll in a ring of cells at the periphery of the Dll domain (Fig. 2B). At the beginning of the third instar, Dll occupies a distinct distal domain and Tsh a proximal one in the disc (Fig. 2C). These territories are separated by 2 to 3 cells in ventral and lateral regions and up to 10 cells in dorsal parts. The Dachshund (Dac) transcription factor is expressed in this intermediate ring of cells overlapping the Dll expression domain by at most 1 or 2 cells (Lecuit and Cohen, 1997). By mid-third instar Dll is expressed in a new, 4-cell-wide, proximal ring that is destined to make the proximal femur and possibly

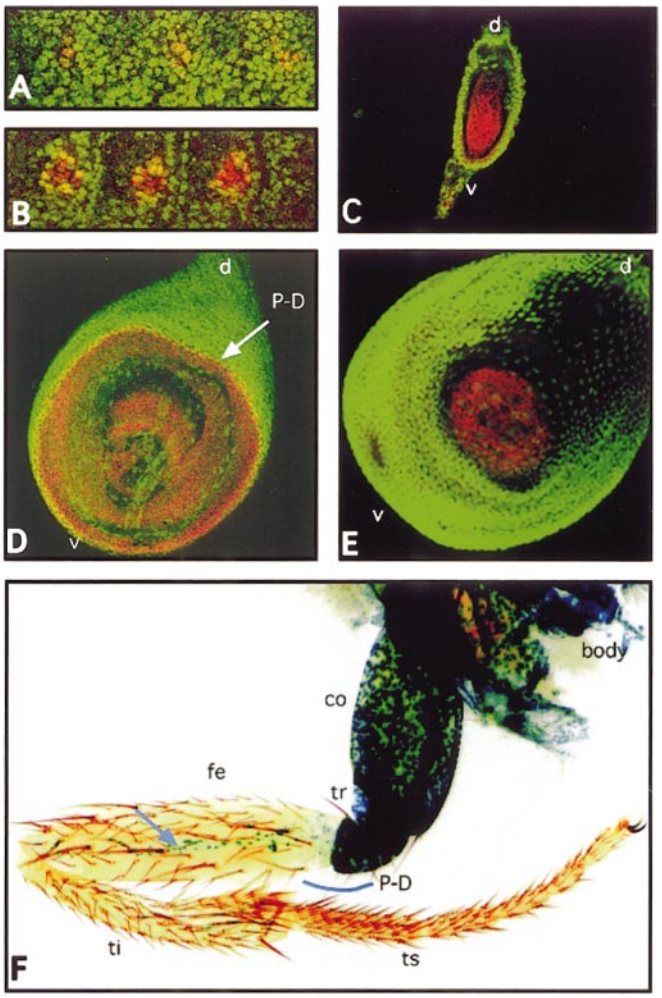

FIG. 2. Overlapping domains of Tsh and Dll proteins during leg development. (A and B) Details of the thoracic hemisegments of *Drosophila* embryos. (A) Tsh (green) is expressed in all cells of the embryonic thorax at stage 10 of embryogenesis. Dll (red) is coexpressed (yellow) with Tsh in the primordia of the leg discs. (B) At stage 15, Tsh and Dll coexpression (yellow) is restricted to a circle at the periphery of the disc primordia, whereas Tsh (green) is expressed alone in the rest of the thorax and Dll (red) in the centre of the primordia. (C) In a early third-instar disc, Tsh and Dll expression domains are separated by two or three cells ventrally (v) and up to 10 cells dorsally (d). Note that the tissue apparently extruding from the ventral side of the disc corresponds to larval structures underneath the disc. (D) A late third-instar disc with focus on the P-D boundary (see Fig. 1A). Tsh (green) is expressed in the proximal domain and Dll (red) in the distal domain. The expression domains overlap (yellow; arrow) by two cells on the P-D boundary. The green cells in the centre of the disc correspond to muscle cells, underlying the basal side of the epithelium. (E) A third-instar disc showing Tsh (green) expression in the peripodial membrane, which covers the apical side of the main epithelium (shown by the red Dll staining) (see Fig. 1A). (F) An adult first leg carrying a LacZ reporter gene located in the *tsh* gene (Fasano *et al.*, 1991), histochemically stained for β-galactosidase (βGal) activity (blue). βGal expression is robust in the coxa (co) and trochanter (tr) and absent from more distal leg parts, including the femur (fe), tibia (ti), and tarsus (ts). Blue staining inside the leg (arrow) indicates muscle cells which derive from the central part of the leg disc but separate from the distal (red) epithelium (see D).

the distal edge of the trochanter (Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999). Tsh overlaps with this new Dll domain at the proximal edge, which persists until the late third-instar stage (Fig. 2D). Additionally, Tsh is still detected in all proximal parts, including the peripodial membrane (Fig. 2E), which overlays the most distal parts of the leg disc (compare with Fig. 1A). Finally in the adult, a Tsh reporter gene is expressed in the body wall, coxa, and trochanter (Fig. 2F). We conclude that different P-D boundaries during leg development coexpress transcription factors required for patterning. In order to simplify the text we use the term "the P-D boundary" to describe the frontier between Tsh-expressing and -nonexpressing cells.

Differential Dorsal–Ventral Effects of Exogenous Teashirt Expression in the Adult Leg

In order to create a new domain of Tsh expression and a thus a novel boundary region, we expressed high levels of Tsh in clones of cells along the P-D axis. In this experiment the transcriptional and posttranscriptional control of *tsh* was uncoupled by expressing *tsh* under the control of two different ubiquitous promoters (*arm* or *actin 5C*), using the *flp*-out technique (Struhl and Basler, 1993, and Materials and Methods). For each promoter the results were similar. Both Tsh-expressing and control wild-type clones were induced at different larval stages and analysed in adult legs (detected with the *yellow*, *y*, cuticular marker). Clones induced at different times during development behaved similarly unless indicated. In control clones, expressing only Gal 4 and not Tsh, *y* clones give wild-type morphologies marking rows of bristles oriented along the P-D axis (Fig. 3A). Control clones appear with a similar frequency in all parts of the leg.

The behaviour of clones expressing high levels of Tsh is different. In the coxa and trochanter, where Tsh is normally expressed, dorsally located *y* clones could integrate into the normal proximal pattern (Fig. 3B, arrows). In contrast, no clones identified by the *y* marker are detected in ventral positions. However, vesicles consisting of bristleless cuticle are formed (Fig. 3C, arrow), suggesting that excess Tsh leads to the disruption of the patterning of this domain. The amount of Tsh seems essential for the patterning of the ventral proximal region, where Wg signalling is active (Fig. 1C).

Clones located distal of but adjacent to the Tsh–Dll boundary in the proximal femur were not observed but vesicles of cuticle develop inside the lumen of the leg, resulting in a reduction of femur length and the perturba-

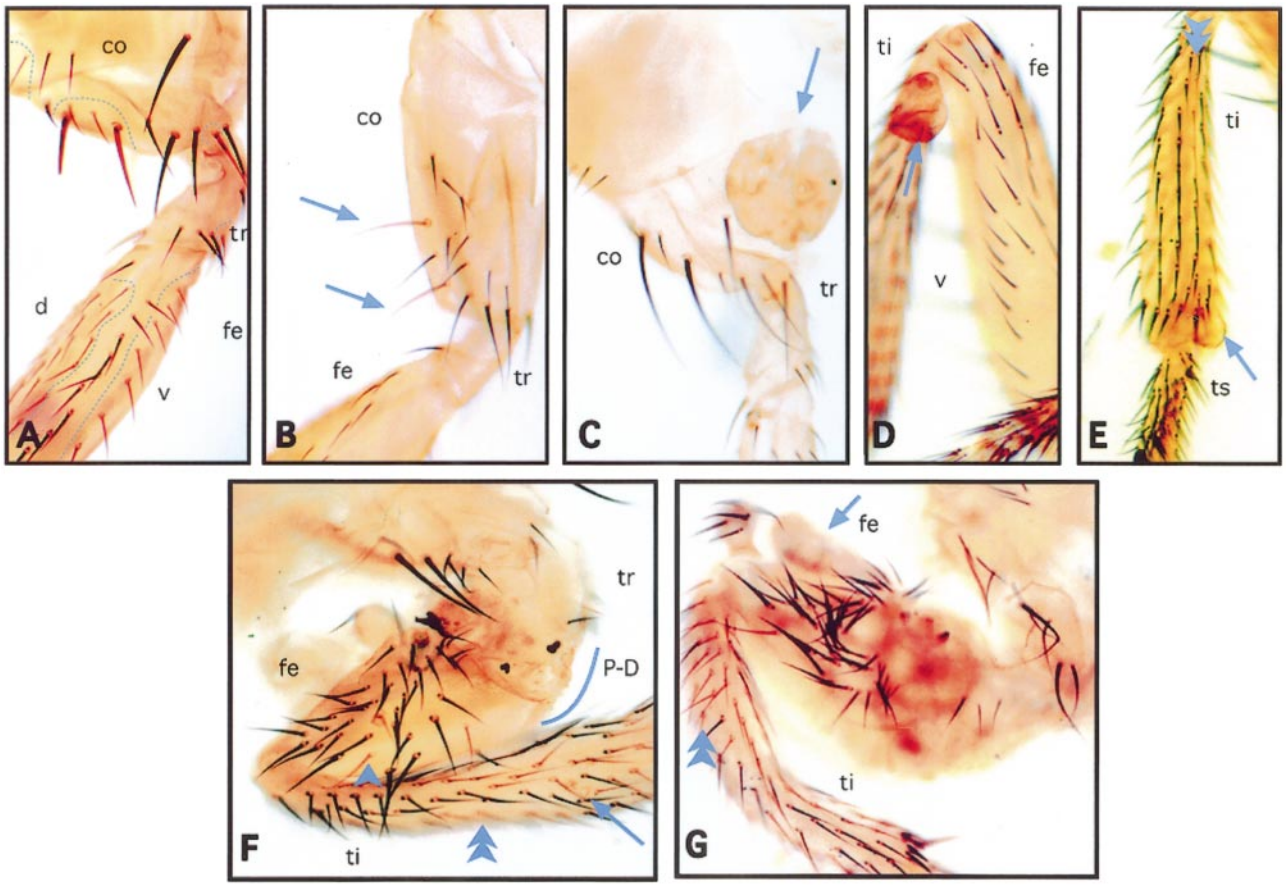

FIG. 3. Differential dorsal–ventral effects of exogenous Tsh production in the adult leg. (A) A second leg with control *y* clones, expressing only Gal4 under *arm/Gal4VP16* control. A dorsal (d) clone is shown crossing from the femur to the coxa. A ventral (v) clone occupies the coxa and the femur. Note that the morphology is normal. (B–G) Cells expressing Tsh under *arm/Gal4VP16* control are detected by the *yellow* (*y*) cuticular marker. Unless indicated, v is on the right. (B) In the dorsal coxa (co), *y* clones survive and are morphologically normal (arrows). (C) In ventral positions in the coxa, vesicles of bristleless cuticle are produced (arrow). (D and E) Vesicle formation induced after longer clone induction by ventrally positioned clones in the tibia (arrows). Note also the normal *y* bristles in a lateral position (double arrowhead in E). (F) Tsh clones (induced at 48–72 h) in the proximal femur disrupt the morphology of the P-D boundary region (blue line, P-D) and lead to defects in patterning. Note for example the nonbracted *y* bristle in the distal femur (arrowhead). *y* clones in the dorsal (double arrowhead) and lateral tibia are morphologically normal. Exceptionally a small vesicle is formed in a lateral clone (arrow). (G) Clones induced at later developmental stages (96–120 h) in the femur cause the development of dorsal outgrowths (arrow) and defects throughout the femur. Lateral and dorsal clones in the tibia develop normally (double arrowhead).

tion of patterning at the boundary region in any D-V position (Figs. 3F and 3G). When clones were induced later in development (in the third-instar larvae), we observed outgrowths especially in dorsal positions (Fig. 3G, arrow). This behaviour is reminiscent of ectopic production of Wg in this position (Struhl and Basler, 1993). In more distal parts of the femur, clones were observed but *y* bristles often lacked bracts (Fig. 3E, arrowhead), similar to loss of *Dll* in this region (Gorfinkiel *et al.*, 1997), which could indicate that these bristles acquire a proximal identity.

In the tibia and tarsus, very few (6/78 legs) *y* clones were detected compared with the numbers of viable proximal ones (45/78 legs), suggesting that cells ectopically expressing Tsh in the distal domain migrate or are lost. The number of surviving *y* clones in distal regions was increased following longer clone induction times (12/84 legs had distal clones after a 10-min heat shock between 48 and 72 h). Clones located in ventral positions are always abnormal, producing fusion between femur and tibia (not shown), vesicles, or outgrowths (Figs. 3C, 3D, and 3E, arrows). However, in lateral or dorsal distal domains, ectopic Tsh clones could fill large regions of the tibia or tarsus with no effect on morphology (see below; Figs. 3E, 3F, and 3G, double arrowheads), except in two cases in which small vesicles of abnormal cuticle were observed to sort out from the leg cuticle (Fig. 3F, arrow).

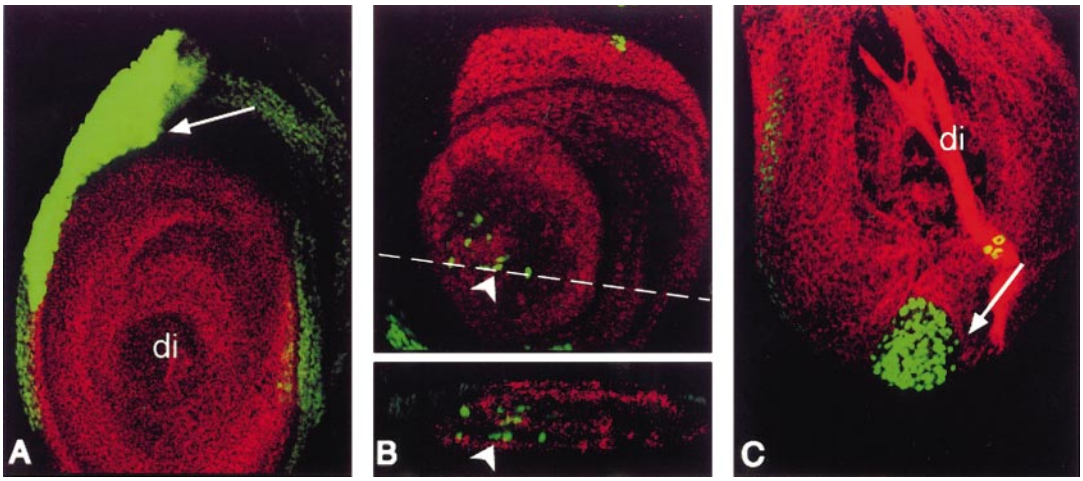

FIG. 4. Exogenous Tsh production in different domains along the P-D axis in leg discs. Ventral is on the bottom. (A) Disc with focus on the P-D boundary. *arm/Gal4VP16*-driven Tsh clones stained for Tsh (green) and Dll (red). A large clone occupies the proximal region (intense green) and forms a straight boundary (arrow) juxtaposed with the Dll-positive cells. Note the absence of Tsh-positive clones in the distal region. (B) Disc (view on the apical side under the peripodial membrane) and cross section showing that isolated cells, expressing high levels of Tsh (green) in the Dll (red) domain, sort out from the disc epithelium (arrowhead). We note that these cells are not muscle cells, since these would lie at a lower plane of focus. (C) Disc with view on the P-D boundary. *Actin 5C/Gal4*-driven clone (arrow) marked by the absence of CD2 (red) after a 10-min induction. This clone extends from the proximal endogenous Tsh domain into the femur region of the disc (compare with Figs. 3F and 3G).

Differential Accumulation of Teashirt in Dorsal and Ventral Domains of the Leg Disc

We next examined the effects of exogenous Tsh production in clones of cells in the third-instar imaginal leg discs (Fig. 4). Clones were detected by the absence of the CD2 cell marker (see Materials and Methods), with a Tsh antibody, or both. We can detect high levels of Tsh in clones within the normal Tsh domain (Fig. 4A) in any D-V or A-P position. These clones often made straight boundaries with Dll-expressing cells (Fig. 4A, arrow), where normally there is no lineage restriction (Steiner, 1976; Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999). This observation supports the idea that Tsh-expressing and -nonexpressing cells have different mixing properties. Whereas we found two or three large clones per disc in the endogenous Tsh domain, mostly isolated cells expressing high levels of Tsh were detected in the distal domain. These cells often sort out from their distal neighbours towards the lumen but remained attached to the epithelium of the disc (Fig. 4B, arrowheads). This behaviour is similar to the effects observed in distal cells lacking *Dll* function or of cells ectopically expressing Dll or Hth, with the difference that these cells sort out as clones and not as isolated cells (Abu-Shaar and Mann, 1998; Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999). However, all of the P-D transcription factors seem to be essential to determining the distinct mixing properties of the cells in which they are expressed.

We then increased the induction time (10 min heat shock) to produce more Tsh-positive cells. As a result there is an higher probability of inducing two or more adjacent Tsh-positive cells. Such Tsh minidomains may be resistant to elimination from the distal domain. We observed groups of Tsh-positive cells that extended into the distal domain (Fig. 4C, arrow). These clones were found in any D-V or A-P position in the border region, accounting for the defects in the adult femur (Figs. 3F and 3G).

Of 50 discs examined, 10 clones expressed Tsh ectopically in the distal domain far from the border region. All these clones occupy ventral positions (Fig. 5A) and correspond presumably to the clones affecting the morphology of the ventral parts of the tibia and tarsus in the adult leg (Figs. 3D and 3E).

Away from the boundary in more distal regions, we have never found large clones of cells which express Tsh ectopically in lateral or dorsal parts, even following longer clone induction times. We induced more clones per disc by a 20-min heat shock, marked by the absence of CD2. Some small clones express Tsh (Fig. 5B, small arrow) but large ones do not (Fig. 5B, arrow). Detection of the *y* marker in these parts of the adult leg (Figs. 3F and 3G, double arrowheads) indicates that the induction system is working, but Tsh protein seems not to accumulate. To test this idea we made ectopic *flp*-out *tsh* clones (induced for 10 min) marked by the coexpression of β-galactosidase (βGal). As before we observed clones of cells expressing high levels of Tsh and βGal in proximal clones. Similarly, in the distal region some isolated cells, expressing both transgenes, were ejected to the lumen of the disc. Whereas coexpression of

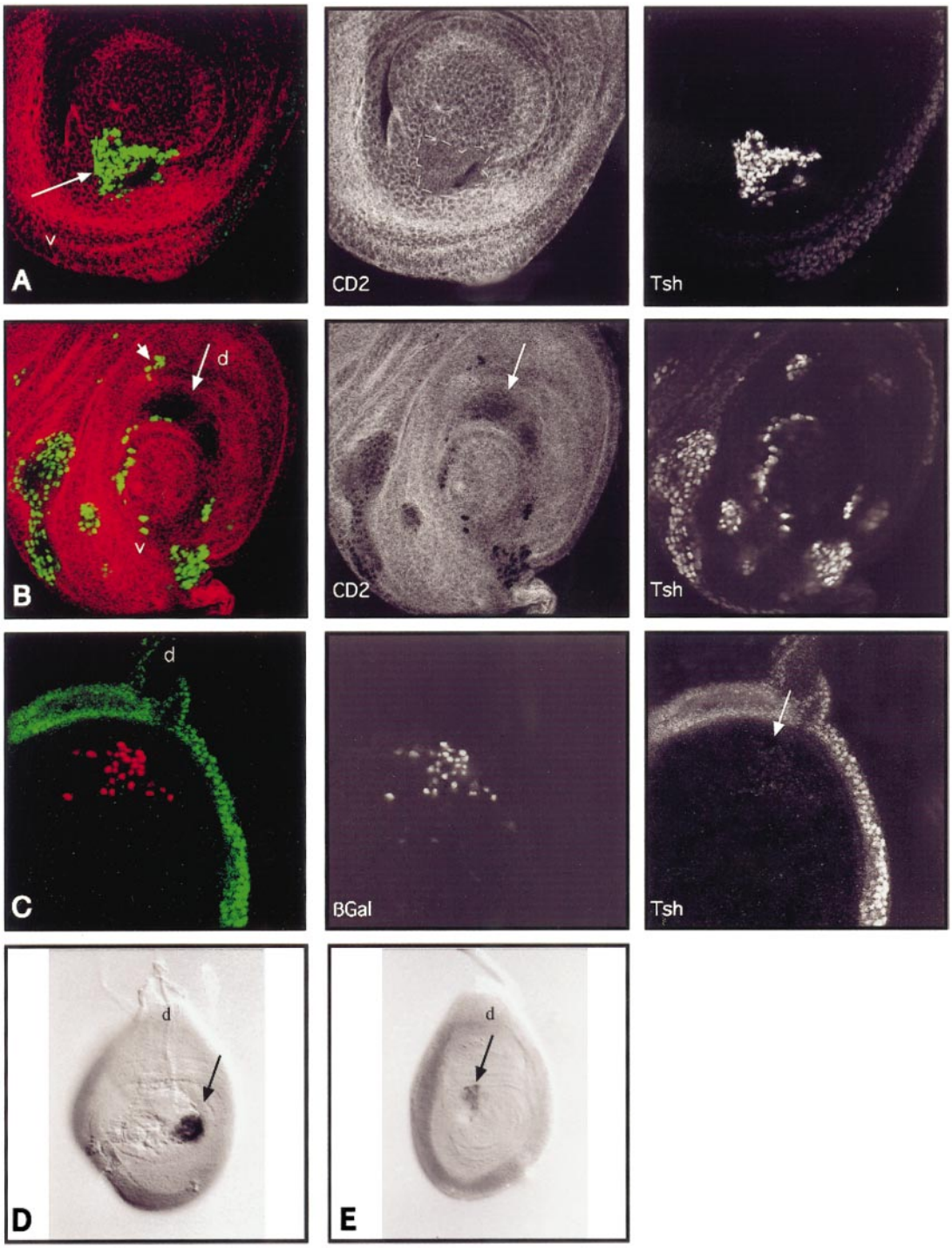

FIG. 5. Differential dorsal (d)–ventral (v) effects of ectopic Tsh expression in the distal leg disc. Clones, indicated by arrows, were induced under *arm/Gal4VP16* control by a 10- (A, C, and D) or a 20- (B) min heat shock. (A) A ventral clone, marked by the absence of CD2 (red and middle) and high Tsh expression (green and right), is integrated into the disc epithelium. (B) Multiple ectopic Tsh clones marked by the absence of CD2 (red and middle). Whereas large proximal and small distal clones (arrowhead) express Tsh (green), larger dorsal, distal clones (arrow) fail to accumulate Tsh. (C) Clone coexpressing *lacZ* and *tsh*. In the dorsal distal domain, βGal (red and middle) is detected at a high level and Tsh is expressed well below the level of endogenous Tsh in the proximal domain (right). (D and E) *In situ* hybridisation to detect *tsh* mRNA in discs after a 10-min heat shock. Unlike Tsh, *tsh* mRNA is detected in clones (arrows) in lateral (D) and dorsal (E) distal positions of the disc.

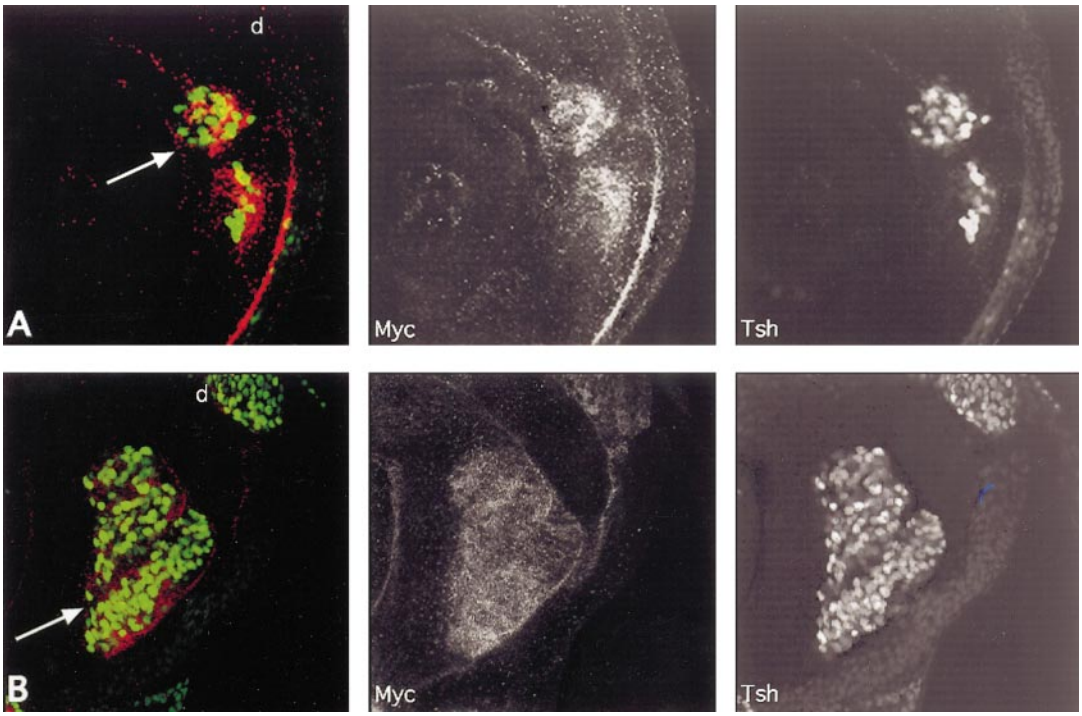

FIG. 6. ArmS10c allows Tsh to accumulate in any dorsal–lateral part of the distal region. Clones coexpressing UASTsh and UASArmS10c were induced by a 5-min heat shock. (A and B) Coexpression of Tsh (green and right) and ArmS10c (red and middle). Whereas Tsh is not detected in distal dorsal or later parts when expressed alone, coexpression with ArmS10c can accumulate Tsh in large clones in these positions.

Tsh and βGal is also detected in the ventral–distal domain (not shown), we found several βGal-expressing clones without, or with very low levels of, Tsh in lateral and dorsal distal regions (Fig. 5C). Consequently large groups of cells expressing the *tsh* transgene are viable in all disc parts, but Tsh protein fails to accumulate to high levels in large clones of cells outside of the ventral distal and the entire proximal territories.

We then made ectopic *flp*-out clones and asked if *tsh* mRNA production is dependent on position. Clones expressing *tsh* mRNA (after a 10-min heat shock) were detected in all parts of the distal domain of the disc, even in lateral (Fig. 5D) and dorsal regions (Fig. 5E). We conclude that the *tsh* transgene is transcriptionally active in all regions of the leg disc, but accumulation of Tsh protein is inhibited in dorsal and lateral parts in the distal domain. As the morphology of the leg in such clones is normal (Figs. 2E and 2F), this effect must be specific to Tsh. That is, βGal is produced (Fig. 5C) and many proteins required for leg morphogenesis are acting normally.

Wingless Signalling Allows Tsh to Accumulate in the Distal Territory

Why should Tsh accumulate in distally located clones only in the ventral region? In this domain Wg is active, allowing Arm to accumulate inside the cells to transmit signalling to the nucleus (Pai *et al.*, 1997; Peifer *et al.*, 1991; reviewed in Wodarz and Nusse, 1998). Arm binds to Tsh both *in vivo* and *in vitro* (Gallet *et al.*, 1998, 1999) and Arm also binds to the cell adhesion protein DE-cadherin (Oda *et al.*, 1994; Orsulic and Peifer, 1996; Pai *et al.*, 1996).

We coexpressed Tsh and ArmS10c, a stabilised form of Arm that constitutively transduces Wg signal (Pai *et al.*, 1997), in the leg disc. Adults or pharate adults were only rarely found in this experiment. When they were, no clones were detected, so we are unable to analyse the effects of Tsh and ArmS10c coexpression after metamorphosis. In the disc, however, whereas ectopic Tsh is rarely detected when expressed alone in dorsal or lateral parts of the distal leg epithelium, ArmS10c allows Tsh to accumulate in groups of cells irrespective of position in the distal domain even following a short (5-min) induction time (Figs. 6A and 6B). Coexpression of Wg and Tsh gives a similar effect (not shown). Since ArmS10c binds to Tsh (Gallet *et al.*, 1998, 1999), we suggest that Wg signalling allows Tsh to accumulate in the distal region.

Teashirt Expression in Dll⁻ Clones

If Tsh can accumulate distally in the Wg domain, why does the P-D boundary form? We wondered whether tran-

scriptional repression may play a role in the maintenance of the P-D boundary. In order to determine whether Dll regulates *tsh*, we removed Dll^+ activity in clones of cells and examined the expression of Tsh in late third-instar leg discs. In the distal part of the leg, Dll^- cells are rejected by their Dll^+ neighbours as shown previously (Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999). As described in Wu and Cohen (1999) Tsh was never detected in surviving Dll^- clones distant from the P-D boundary, indicating that Dll is not sufficient to explain the negative regulation of *tsh* in the distal domain. However, close to the P-D boundary, Tsh was expressed ectopically in cells lacking Dll^+ activity in the ventral region (Fig. 7A, arrow). Dll^- clones in the lateral and dorsal parts close to the boundary did not express Tsh (Fig. 7A, arrowhead). Part of the mechanism for boundary formation is therefore a repression of *tsh* by Dll in ventral cells, where Wg signalling is active.

Reduced Teashirt Function in the Leg

Does Tsh have a function in the proximal domain and particularly for boundary formation? We have made new alleles of *tsh*, which are semiviable and affect the proximal part of the leg. In tsh^3 hemizygotes the coxa and trochanter are fused together and a part of the proximal femur is deleted (Fig. 7B; compare to a wild-type leg in Fig. 2F). A stronger allele, $tsh^{12\text{-}5}$, is lethal as a hemizygote, with individuals dying at different times during pupation. Analysis of the legs from pharate adults shows that large deletions of cells occur from the boundary region (Fig. 7C). Loss of Tsh activity seems to affect the cells on either side of the boundary (trochanter and femur), whereas an enhancer trap insertion in the *tsh* gene (Fasano *et al.*, 1991) is expressed only in the trochanter and coxa of the adult legs (Fig. 1F). Bracted bristles, typical of the distal leg, replace the proximal patterns and exhibit reversed polarity, with respect to the normal distal leg patterns (Fig. 7D, arrow). These observations show that Tsh is especially critical for proximal domain identity but also contributes to P-D boundary formation.

We examined Tsh and Dll expression in these *tsh* mutations. Tsh is still detected in tsh^3 (not shown) and $tsh^{12\text{-}5}$ hemizygotes, but strikingly the Tsh–Dll boundary occupies a more proximal location in the peripodial membrane where normally Dll is never detected (Fig. 7E, arrowhead). The ring of cells coexpressing Tsh and Dll is at least 10 cells wide, instead of at most 2 in wild type (Fig. 2D). The proximal boundary of expression of *dac*, another gene expressed in a localised region along the P-D axis (Fig. 7G; Mardon *et al.*, 1994; Lecuit and Cohen, 1997), is also located more proximally in these weak *tsh* mutations (Fig. 7F). Normal Tsh activity seems to be required for the correct location of the P-D boundary, by determining proximal border of Dll and Dac expression.

DISCUSSION

We have examined the role of Tsh for leg patterning in *Drosophila*. Tsh expression is restricted to a proximal ring and the peripodial membrane (Fig. 2; Gonzalez-Crespo and Morata, 1996), destined to make the body wall, coxa, and trochanter (Fig. 2F). We show that Tsh plays a role in proximal leg morphogenesis and boundary formation between Tsh-expressing and -nonexpressing cells, as hypomorphic mutants affect not only the development of the proximal leg parts (Fig. 7) but also the morphology of the region just adjacent to Tsh-expressing cells. We have analysed an artificial situation by expressing exogenous Tsh in clones (Figs. 3–6). Surprisingly we found that such clones exhibit differential D-V effects in the developing leg. In dorsal and lateral regions Tsh protein cannot accumulate in such clones, suggesting that a posttranscriptional mechanism allowing the maintenance of the P-D boundary in the absence of Wg signalling exists. In cells in which Wg is signalling, Tsh protein can accumulate in the distal domain. Dll, however, acts as a repressor of *tsh* in Wg active cells, which keeps the boundary intact in ventral positions.

Differential Mixing Properties between Proximal and Distal Regions

By ectopically expressing Tsh distally, we show that cells expressing Tsh sort out from the distal domain (Fig. 4B). In the endogenous Tsh-expression domain, cells overexpressing Tsh divide normally and form clones which mix perfectly with the neighbouring proximal cells. However, these clones made straight boundaries with distal cells (Fig. 4A) despite the fact that there is no lineage restriction along the P-D axis (Steiner, 1976; Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999). These observations indicate that proximal and distal cells have different mixing properties. In normal development, cells with these distinct mixing properties confront one another only at the P-D boundary.

Similar results have been described by Abu-Shaar and Mann (1998), Campbell and Tomlinson (1998), and Wu and Cohen (1999), who showed that Dll and Hth are required for the particular adhesion properties of the distal and proximal domains, respectively. Loss or ectopic expression of these transcription factors causes these mutant cells to sort out from their surrounding wild-type neighbours. Thus localised transcription factors are responsible for the particular adhesion properties of the cells in which they are expressed. However, at the moment it is not clear how these transcription factors regulate cell mixing. Tsh might regulate a particular set of target genes giving proximal adhesion characteristics, whilst other transcription factors (e.g., Dll or Hth) will determine distinct mixing properties, allowing different cell populations to remain separated in a stable and heritable manner.

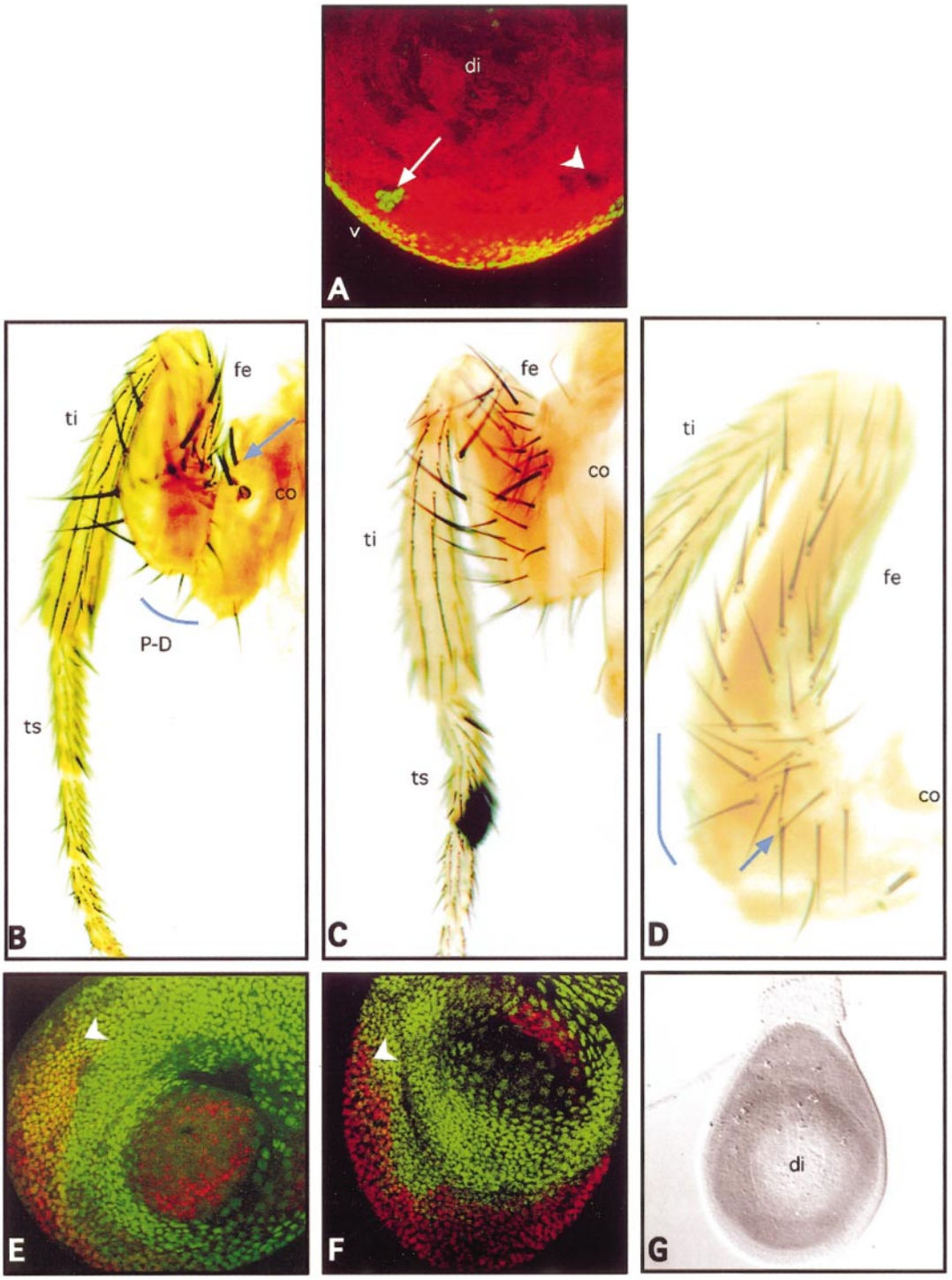

FIG. 7. Mutual repression between Tsh and Dll is critical for boundary formation in ventral cells. (A) *Dll*$^{-}$ clones were induced at 72–120 h after egg laying and are marked by the absence of βGal (red). Ventrally (v), Tsh (green) is ectopically expressed in clones close to the endogenous Tsh domain (arrow). Clones in other regions do not express Tsh ectopically (arrowhead). (B, C, and D) Hypomorphic loss of *tsh* affects P-D boundary formation as well as bristle polarity (arrow) and identity in the proximal domain. (B) A *tsh*^{3}*/Df(2L)305* adult first leg. Note the normal distal parts but a reduced region around the P-D boundary; the trochanter and coxa regions are fused and the femur is reduced in size (compare with Fig. 2F). (C) *tsh*$^{12\text{-}5}$*/Df(2L)305* legs showing a more severe reduction of the coxa, trochanter, and proximal femur region. (D) A *tsh*$^{12\text{-}5}$*/Df(2L)305* second leg with fusion of the proximal structures. Note that nonbracted bristles, typical for the

Tsh and Dll Combine with Wg Signalling Activity for Transcriptional Regulation between Proximal and Distal Domains

The maintenance of the P-D boundary presumably depends on transcriptional regulation between different P-D factors, as is the case for the A-P compartments of limbs (Blair and Ralston, 1997; Garcia-Bellido, 1975). Recently conflicting reports concerning the cross regulation between different P-D factors have appeared (Abu-Shaar and Mann, 1998; Gonzalez-Crespo *et al.*, 1998; Wu and Cohen, 1999). In distally located Dll^- clones, Wu and Cohen (1999) found that Hth was not ectopically expressed, contrary to the reports of the other groups. In similar Dll^- clones we found that Tsh was expressed ectopically only in the ventral part close to the boundary region (Fig. 7A). The ventral leg relies on information provided by the Wg signal transduction pathway. Inside of these cells, Arm is stabilised and associates with the *Drosophila* T Cell factor to regulate target genes (reviewed by Wodarz and Nusse, 1998). Our results support the idea that Dll represses *tsh* expression in parts of the leg disc where Wg is signalling. In accord with this idea ectopic Tsh disrupts the patterning of the leg in any P-D location in Wg signalling receiving cells (Figs. 3C–3E), whereas in other parts of the leg, ectopic *tsh* has no effect on patterning (Figs. 3B, 3F, and 3G). In the leg, we suggest that mutual repression between P-D factors is of particular importance in ventral cells. Recently we have shown that Tsh is able to associate *in vitro* and *in vivo*, in the embryo, with Arm (Gallet *et al.*, 1998, 1999). The stability of Tsh in the ventral distal parts of the leg disc could be due to the interaction between Tsh and Arm. In fact, Tsh does not accumulate ectopically outside of the Wg signalling domain in the distal region. However, coexpression with Arm allows Tsh to accumulate in any distal position (Fig. 6). In conclusion, our results suggest that P-D transcription factors may act in combination with Wg signalling for the maintenance of P-D boundaries.

Boundary Maintenance in the Absence of Wg Signalling

Mutual repression between the P-D transcription factors does not seem to be responsible for boundary maintenance where Wg signalling is inactive (Fig. 7A). How is the boundary maintained in the absence of mutual repression? Clones ectopically expressing *tsh* outside the Wg signalling domain do not accumulate Tsh protein, whereas clones in the Wg-receiving cells do (Fig. 5). Accumulation of Tsh seems to be inhibited outside of the Wg active domain. This may be relevant to the maintenance of the cell-lineage-independent P-D boundaries of the legs. We suggest that cells expressing Tsh outside of its normal domain (just distal to the P-D boundary) are recognised by the surrounding distal cells due to their distinct adhesion properties. This results in the inhibition of the accumulation of Tsh protein specifically in the cells distal to the boundary. In accord with this idea, ectopic Tsh cannot accumulate in dorsal clones distal to the P-D boundary. Ectopic Wg signalling, however, favours the accumulation of Tsh in the distal domain. Arm binds to Tsh (Gallet *et al.*, 1998), raising the possibility that binding stabilises Tsh in cells and changes their adhesion properties, allowing them to integrate into the epithelium. Despite the differences in mixing, cells can divide and straddle P-D boundaries (Gorfinkiel *et al.*, 1997; Wu and Cohen, 1999), implicating the need for such a mechanism to eliminate localised factors when misplaced from their normal domain. This mechanism will ensure that boundaries and domains are maintained independent of Wg signalling.

At the present time we do not know the precise molecular mechanism preventing Tsh accumulation in ectopic positions. At the P-D boundary, cells express both Tsh and Dll (Figs. 1B and 1D), and at other boundaries along the P-D axis, coexpression of other localised transcription factors has been observed (Gonzalez-Crespo and Morata, 1996; Lecuit and Cohen, 1997; Rieckhof *et al.*, 1997; Wu and Cohen, 1999). We propose that when a boundary cell occupies a more distal (or proximal) position, cell interactions prevent the accumulation of the transcription factor localised in cells occupying an ectopic position with respect to the boundary. Such a mechanism would ensure the maintenance of P-D boundaries in the absence of cell lineage restrictions.

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proximal leg, are replaced by bracted bristles with reversed polarity (arrow). (E) A $tsh^{12\text{-}5}/Df(2L)305$ third-instar leg disc stained for Dll (red) and Tsh (green). Tsh is still expressed but Dll extends proximally into the peripodial membrane of the disc; compare with expression in a wild-type disc (Fig. 2E). Dll and Tsh are coexpressed (arrowhead) in more cells in the ventral part of the disc. (F) A $tsh^{12\text{-}5}/Df(2L)305$ leg disc showing DaclacZ (red) and Tsh (green) expression. DaclacZ is ectopically expressed in the proximal region. In a normal disc, DaclacZ expression is never seen at this focal plane. (G) X-Gal staining from the dac^P line, reflecting the endogenous *dac* expression. DaclacZ is expressed in an intermediate region and is excluded from the most distal part (di) and from the peripodial membrane, the coxa, and the trochanter.

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