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Development of the *Drosophila* genital disc requires interactions between its segmental primordia

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SUMMARY

In both sexes, the *Drosophila* genital disc comprises three segmental primordia: the female genital primordium derived from segment A8, the male genital primordium derived from segment A9 and the anal primordium derived from segments A10-11. Each segmental primordium has an anterior (A) and a posterior (P) compartment, the P cells of the three segments being contiguous at the lateral edges of the disc. We show that Hedgehog (Hh) expressed in the P compartment differentially signals A cells at the AP compartment border and A cells at the segmental border. As in the wing imaginal disc, cell lineage restriction of the AP compartment border is defined by Hh signalling. There is also a lineage restriction barrier at the segmental borders, even though the P compartment cells of the three segments converge in the lateral areas of the disc. Lineage restriction between segments A9 and A10-11 depends on factors other than the Hh, En and Hox genes. The segmental borders, however, can be permeable to some morphogenetic signals. Furthermore, cell ablation experiments show that the presence of all primordia (either the anal or the genital primordium) during development are required for normal development of genital disc. Collectively, these findings suggest that interaction between segmental primordia is required for the normal development of the genital disc.

Key words: *Drosophila* genital disc patterning, Hh signalling, Dpp signalling, Wg signalling, Segmental boundaries, Cell lineage restriction

INTRODUCTION

The development of *Drosophila* imaginal discs to a large extent depends on the genetic interactions that take place at the border between anterior (A) and posterior (P) cells. These two populations of cells differ as the selector gene *engrailed* (*en*) is expressed in P cells but not in A cells. The limit between A and P cells is the compartment border and acts as a source of positional information (reviewed by Lawrence and Struhl, 1996). En activates the diffusible signal Hedgehog (Hh) in P cells, which signals to A cells and activates two different morphogens, Wingless (Wg) and Decapentaplegic (Dpp) (Basler and Struhl, 1994). Wg and Dpp act as general organisers of the imaginal discs, inducing growth and specifying different identities in a concentration-dependent manner (Zecca et al., 1996; Lecuit and Cohen, 1997). This model has arisen mainly from studies performed on the wing and leg discs. These are single discs derived from a cluster of cells of a unique segment in the embryo (Bate and Martínez-Arias, 1991).

The genital disc, which gives rise to the terminalia of the adult fly (internal and external genitalia excluding the gonads and analia) (Bryant, 1978), is organised in a manner reminiscent of the leg disc (Gorfinkiel et al., 1999; Estrada and Sánchez-Herrero, 2001). However, there are several differences between the genital disc and other imaginal discs that make it an original experimental model (reviewed by Sánchez and Guerrero, 2001). For example, it is the only imaginal disc that shows clear sexual dimorphism. The genital disc is a compound disc formed by the fusion of three different primordia derived from the embryonic abdominal segments 8, 9 and 10-11 (Nöthiger et al., 1977; Schüpbach et al., 1978; Dübendorfer and Nöthiger, 1982; Epper and Nöthiger, 1982). Thus, a genital disc contains a female genital primordium (derived from segment A8), a male genital primordium (derived from segment A9) and an anal primordium (derived from segments A10-11). In females, the female primordium gives rise to almost all the internal and external genitalia. The male primordium was considered to be a 'repressed primordium' because its growth is limited and it does not form any adult structures (Epper and Nöthiger, 1982). However, it has recently been shown that the male primordium in the female genital disc gives rise to the parovaria (internal accessory female glands) (Keisman et al., 2001). The anal primordium in females gives rise to the female analia. The converse is true for males. In male genital discs, the male genital primordium gives rise to the majority of the internal and external male genital structures. The female genital

primordium was also considered to be a repressed primordium but it is now known to produce a miniature eighth tergite (Keisman et al., 2001). The anal primordium in the male genital disc gives rise to the characteristic male anal plates.

Contrary to the situation in other imaginal discs, in the genital disc, the response to Hh, Dpp and Wg is controlled by the sex determination genes (Sánchez et al., 2001; Keisman and Baker, 2001). The last gene in the sex determination cascade is *doublesex* (*dsx*), which encodes two zinc finger proteins, Dsx^M in males and Dsx^F in females. In the female genital discs, Dsx^F blocks the transcription of *dpp* induced by Hh in the A9, while in the male genital disc, Dsx^M modulates the response to Wg in A8 cells. This results in the differential growth and development of the genital primordium of the corresponding sex and its respective 'repressed primordium'.

Apart from the additional control exerted by the sex determination genes in the genital disc, the fact that three different segments develop side by side is a further characteristic feature of genital discs that distinguishes them from other imaginal discs. Each primordium of the genital disc has an anterior (A) and a posterior (P) compartment (Freeland and Kuhn, 1996; Casares et al., 1997; Chen and Baker, 1997). The situation is thus similar to that in the embryo and in the abdomen, where P cells from one segment come into contact with A cells located anteriorly at the AP compartment border, and with A cells located posteriorly at the PA segmental border. Extensive work on the abdomen has shown that Hh signals to A cells both for and aft, and that these cells respond differently to the Hh signal (Struhl et al., 1997a; Lawrence et al., 1999). Hh induces *optomotor-blind* (*omb*) expression in cells at the AP border but not at the segmental border (Kopp and Duncan, 1997). In the embryo, Hh induces Wg expression in cells at the AP border, while no Wg expression is observed at the PA border (O'Keefe et al., 1997; Sanson et al., 1999).

It has been shown that both AP and PA boundaries act as cell lineage restriction borders, although in the embryo, PA restriction occurs later than the AP restriction (Vincent and O'Farrell, 1992). Cell lineage restriction studies have shown that an interface between En/Hh and non-En/Hh expressing cells is required to form such boundaries (García-Bellido et al., 1973; Kornberg, 1981; Rodríguez and Basler, 1997; Blair and Ralston, 1997). In the genital disc, a cell lineage restriction border was found between the genitalia and analia, indicating that a segmental boundary exist between A8/A9 and A10-11 (Dübendorfer and Nöthiger, 1982). It has also been shown that by the beginning of the second instar, or even earlier, the genital disc has already subdivided into anterior and posterior compartments (Chen and Baker, 1997).

We have reanalysed the presence of cell lineage restriction borders in the genital disc and address the question of whether segmental borders (the borders between the different primordia) differ from compartment borders. We have also looked for communication between the different primordia and explored whether this communication is required for the development of the genital disc. The results obtained prompt our reconsideration of genital disc organisation, and we propose a situation in which the three primordia develop interdependently.

MATERIALS AND METHODS

Fly stocks

The reporter genes *dpp-lacZ* (Blackman et al., 1991) and *hh-lacZ* (Heberlein et al., 1993) are expressed as the endogenous RNA in the genital disc (data not shown). The following GAL4 drivers were used for the ectopic expression experiments (Brand and Perrimon, 1993): *en-Gal4* (Brand and Perrimon, 1993), *cad-Gal4* and tsh-GAL4 (Calleja et al., 1996). We used the *act>lacZ; actin>β-gal* stock (Struhl and Basler, 1993) to induce ectopic *lacZ* clones. The UAS-*dpp* and UAS-*hh* are described elsewhere (Capdevila and Guerrero, 1994). The UAS-RicinA and the cold-sensitive Ricin mutant protein (RAcs2) have been described previously (Smith et al., 1996; Moffat et al., 1992). The dpp^{d5}/dpp^{d12} mutant combination shows alterations in the disc regulatory region of the *dpp* gene (St Johnston et al., 1990). For the ectopic expression of *dpp*, *tub > y+ > dpp* chromosome was used (Sánchez et al., 1997). The heat-shock flipase used was *FLP122* (Struhl and Basler, 1993). The *wg* mutant combinations were wg^{CX3}/wg^{IL114} at 29°C or wg^{CX3}/Sp. *Sternopleura* is a regulatory mutation of *wg* with both dominant and recessive effects. The loss-of-function component (wg^{CX3}/Sp mutant combination) reflects a reduction of *wg* activity in the notum and in the antenna (Neumann and Cohen, 1996). We found that it also affects the male and female genitalia.

Flies used for the analysis of external terminalia of adults were kept in a mixture of ethanol:glycerol (3:1) for several days. They were then macerated in 10% KOH at 60°C for 15 minutes, thoroughly washed with H_2O, and mounted in Faure's solution for examination under a compound microscope.

Clonal analysis

Clones of mutant cells were generated by FLP-mediated mitotic recombination (Golic, 1991) and lineage clones were generated using the flip-out GAL4 system (Pignoni and Zipursky, 1997). For the generation of *smo* and *smo cad* clones, *yFLP122; smo FRT 40, hh-lacZ* or *yFLP122; smo cad FRT40* females were crossed to *GFP FRT40* males and 24-72 hour larvae were heat shocked for 60-90 minutes. For the generation of lineage clones in a dsx^1 background, *act>>lacZ;* dsx^1*/SM5;TM6B/+* females were crossed to *yFLP122;* dsx^1*/SM5, TM6B/+* males and 12-24 hour embryos were heat-shocked for 10 minutes. Lineage clones were also generated crossing *act>>GAL4 UAS-GFP* females with *HmcFLPIII* males and 12-24 hour embryos were heat-shocked for 10 minutes.

Antibody staining

The antibodies used were the following: monoclonal anti-En 4D9 (Patel et al., 1989); polyclonal anti-Cad (Macdonald and Struhl, 1986); anti-Ptc antibody ApaI (Capdevila et al., 1994); monoclonal anti Abd-B 1A2E (Celniker et al., 1989); monoclonal anti-Wg (Brook and Cohen, 1996); anti-Tsh antibody (S. Kerridge); anti-Dll antibody (Vachon et al., 1992); monoclonal anti-MYC 9E10 (Babco, Berkeley antibody company); and anti-β-gal (Jackson Laboratories).

RESULTS

Hh signalling in the genital disc

The segmental and compartmental organisation of the genital disc is schematically shown in Fig. 1. The parasagittal section clearly shows that the three primordia of the genital disc are contiguous. This means that the P compartment of one segment is adjacent to the A cells of the corresponding segment, and to the A cells of the following segment. Hh is expressed in the three P compartments. To establish whether Hh is able to signal in both directions, as occurs in the embryo (Heemskerk and

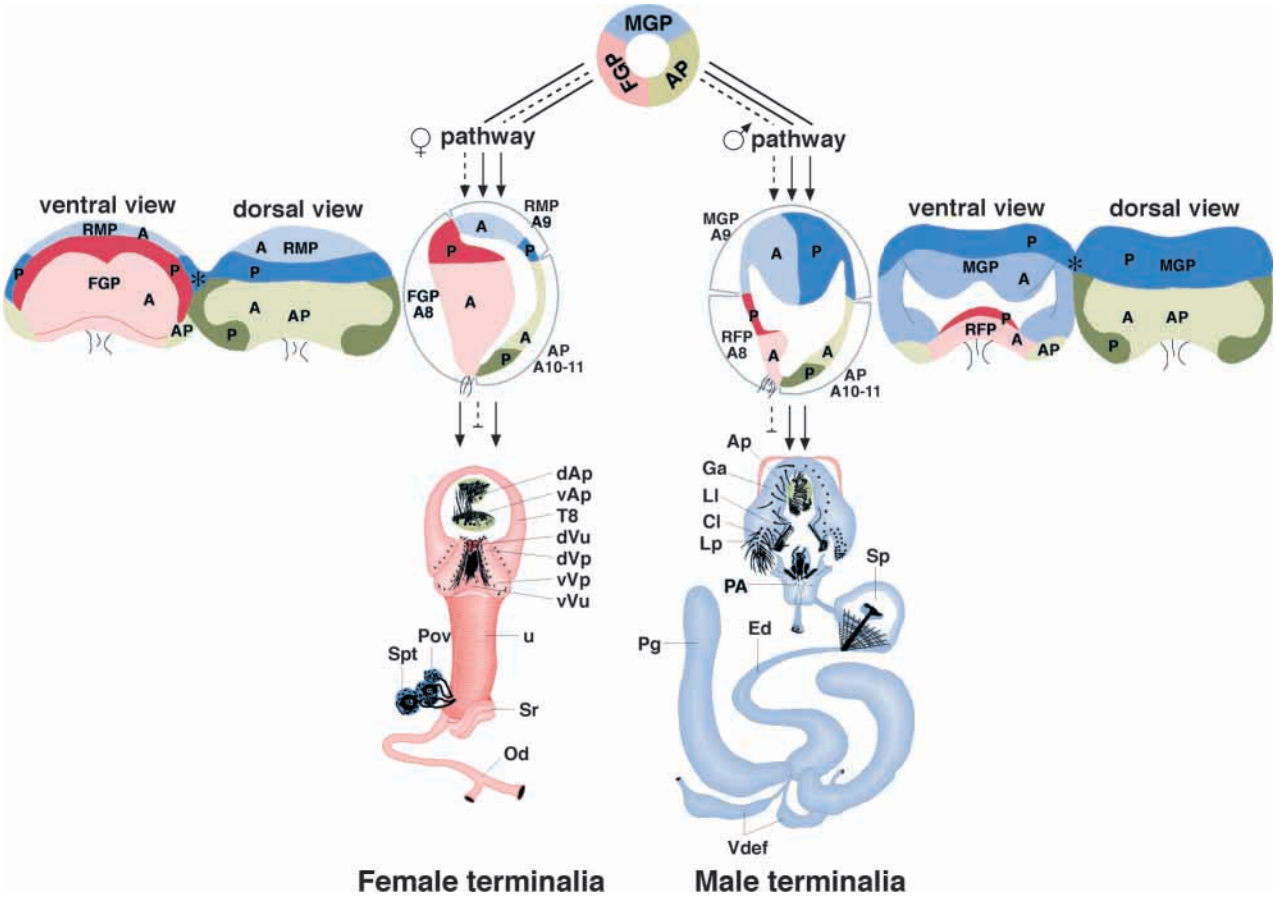

Fig. 1. Development of the genital disc and adult derivatives in both sexes. The female and male genital discs are shown as ventral, dorsal and parasagittal views. A8 (female primordium, pink), A9 (male primordium, blue) and A10-11 (anal primordium, green) indicate the corresponding abdominal segments. The posterior compartment of each primordium is shown in darker colours (pink, blue and green). Note that each posterior compartment is adjacent to the anterior cells corresponding to its own segment and to the anterior cells of the following segment, as occurs in the abdomen (parasagittal view). The P compartment cells of the three segmental primordia converge in the lateral areas (asterisk) but not in the centre of the disc. A, anterior compartment; P, posterior compartment; FGP, female genital primordium; MGP, male genital primordium; AP, anal primordium; RFP, repressed female genital primordium; RMP, repressed male genital primordium. External female terminalia: dAp, dorsal anal plate; vAp, ventral anal plate; T8, tergite eight; dVu, dorsal vulva; vVu, ventral vulva; dVp, dorsal vaginal plate; vVp, ventral vaginal plate. Internal female terminalia: U, uterus; Sr, seminal receptacle; Spt, spermatheca; Pov, parovaria; Od, oviduct (connected to the ovaries). Male external terminalia: Ap, anal plate; Ga, genital arch; Ll, lateral lobe; Lp, lateral plate; Cl, clasper; PA, penis apparatus. Internal male terminalia: Ed, ejaculatory duct; Sp, sperm pump; Pg, paragonia (male accessory gland); Vdef, vas deferens (connected to the testes). Modified, with permission, from Epper and Nöthiger (Epper and Nöthiger, 1982) and P. C. Ehrensperger (PhD thesis, University of Zürich, Switzerland, 1983).

DiNardo, 1994) and abdomen (Struhl et al., 1997a; Struhl et al., 1997b), we examined the expression of the Hh receptor, Patched (Ptc) (Hooper and Scott, 1989; Nakano et al., 1989). If the Hh pathway is active, upregulation of Ptc expression should be observed on both sides of Hh-expressing cells. Accordingly, we detected two bands of Ptc expression flanking each P compartment (Fig. 2A-D). Previous reports only attempted to show the anterior band of Ptc expression and failed to observe the band of Ptc expression in A cells of the following segment (Freeland and Kuhn, 1996; Casares et al., 1997; Chen and Baker, 1997). This could be best seen at the boundary between segments A9 and A10-11 both in males and females (Fig. 2B,D). Note that at the P compartment of segment A9, a region of Ptc expression was observed in anterior A9 cells (Fig. 2D, arrowhead), and another, narrower, band of Ptc was detected posteriorly towards the anal primordium (Fig. 2B,D, arrow). That this novel band of Ptc expression was indeed in the anal primordium was confirmed by genital discs doubly stained by Ptc and Caudal (Cad) expression (Fig. 2E-G). The latter gene is specifically expressed in the analia (Moreno and Morata, 1999). We conclude that in the P compartment of A9 (male genital primordium), Hh signals both A9 and A10-11 (anal primordium).

In male genital discs, the A8 segment is a small mass of cells located at the ventral posterior end of the disc (Fig. 1) and corresponds to the ‘female repressed primordium’ (RFP) with A and P compartments, the latter being contiguous to the A compartment of the A9 segment (the male genital primordium, MGP). We also observed two bands of Ptc expression flanking the P cells of the small A8 segment in male genital discs, one corresponding to the anterior cells of the A8 (Fig. 2A,

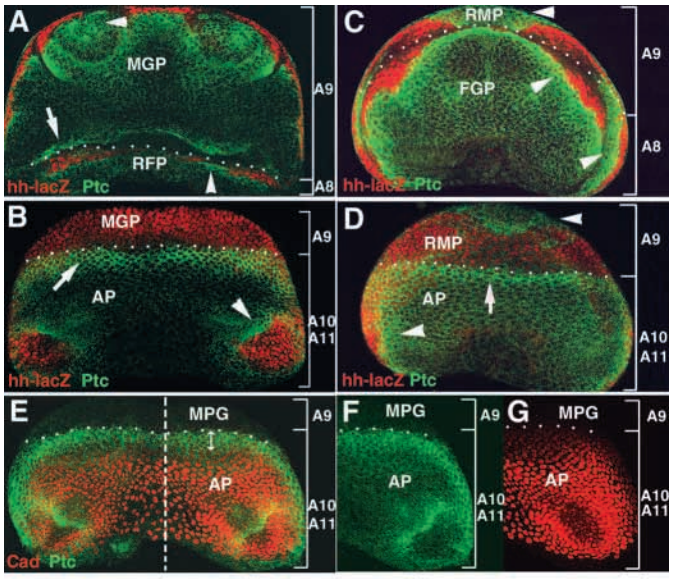

Fig. 2. Hh from posterior compartment cells signals to anterior cells both infront and behind as shown by Ptc expression in male and females. (A-D) Ptc (green) and *hh-lacZ* (red) expression in male (A,B) and female (C,D) genital discs. The segmental primordia corresponding to each genital primordium are indicated on the right side of each panel. The broken line separates the different genital primordia in each disc. Expression of *ptc* induced by Hh at the segmental border (arrows) and parasegmental borders (arrowheads). (E) Expression of Cad (red) and Ptc (green) in the A10-11 segment. In the analia, Cad is expressed in a gradient (double arrows) in the most anterior part of the A10-11 segment. (F,G) This new band of *ptc* expression (F) overlaps with the lowest intensity level of the *cad* expression gradient (G) in the analia.

arrowhead), and the other, to a narrow band of cells in A9 (Fig. 2A, arrow).

In female genital discs, the border between segments A8 and A9 was more difficult to detect because of complex folding of the disc. We were unable to distinguish two different bands of Ptc expression flanking the A8 posterior compartment, probably because the new band of Ptc expression induced in A9 by posterior A8 cells fuses with the 'normal' Ptc-expressing cells at the A9 AP compartment border (Fig. 2C).

These observations led us to question whether anterior cells respond differently to the Hh signal depending on their position with respect to the Hh source, as occurs in the embryo and abdomen (Heemskerk and DiNardo, 1994; O'Keefe et al., 1997; Struhl et al., 1997a; Alexandre et al., 1999). To address this question, we concentrated on analysing the segmental border between segments A9 (male genital primordium) and A10-11 (anal primordium), owing to its improved visibility over the A8/A9 border, both in male and female genital discs. As previously shown, in male genital discs Hh signals anterior A9 cells, inducing the activation of Wg and Dpp in mutually exclusive domains (Chen and Baker, 1997; Sánchez et al., 1997; Gorfinkiel et al., 1999). In female genital discs, anterior A9 cells respond to Hh signal, activating Wg only; Dpp activation was not observed because the sex-determination gene Dsx^F blocks its transcription in response to Hh (Sánchez et al., 2001). We then went on to determine how the anterior A10-11 cells responded to Hh emerging from posterior A9 cells. No expression of Wg or Dpp was observed (data not shown).

We have observed that *en* and *hh* expression domains did not fully coincide in posterior A9 cells (Fig. 3A). At the anterior border of the A9 posterior compartment, En and *hh-lacZ* colocalised and their limit of expression appeared as a straight edge. By contrast, at the posterior border of A9, the limits were more diffuse, and the En expression domain extended beyond the *hh-lacZ* domain in the anterior region of segments A10-11 (Fig. 3B). To establish whether the segmental border corresponds to the posterior border of the Hh expression domain or to that of En expression, we stained genital discs for Cad and En. Surprisingly, we found that there was a narrow band of cells where Cad and En overlapped (Fig. 3C,D). This indicated that En was expressed in anterior A10-11 cells. This situation resembles that of the AP compartment border of wing imaginal discs, where En is expressed in anterior cells (Blair, 1992; Hidalgo, 1994) in response to Hh (Guillén et al., 1995; de Celis and Ruiz-Gomez, 1995) in late third instar larval imaginal discs. We also noted that the limit of Cad expression was not well defined. On the contrary, Cad levels were downregulated at the A9/A10-11 border (Fig. 3D). To determine if these properties of the A9/A10-11 border (i.e. Ptc expression, En expression and Cad downregulation) were due to a response to the Hh signal, we experimentally altered Hh levels. To increase Hh levels, we analysed genital discs from *en-GAL4/UAS-hh* flies. In these discs, we observed expanded Ptc and En expression domains in anterior A10-11 cells abutting the A9/A10-11 segmental border (Fig. 4A).

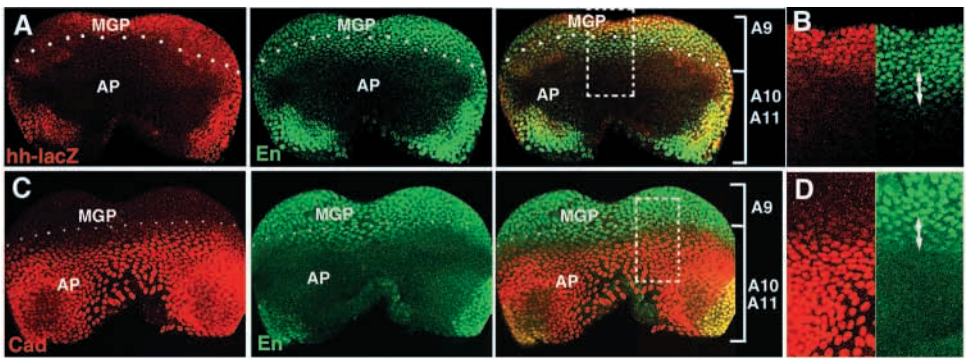

Fig. 3. En expression in the posterior A9 extends to the anterior A10-11. (A) En (green) and Hh (red) expression in A9 and A10-11 segmental primordia (AP) in the male genital disc. En expression in the posterior compartment of A9 segment extends to the A10-11 segment in a graded manner (double arrow, B). (C) En (green) and Cad (red) expression in A9 and A10-11 segmental primordia (AP) in the male genital disc. En and Cad expression overlap in the most anterior cells of the A10-11 segment. A gradient of both En and Cad expression is observed (double arrow) in the overlapping region (D). Hh expression therefore defines the border between segments A9 and A10.

Consistently, the region where Cad levels were downregulated was also extended (Fig. 4B).

To test if Hh signal crosses the segmental border to adjacent A cells of the following segment, homozygous null clones for *smoothened* (*smo*) were induced. Smo is a serpentine transmembrane protein required for Hh signal transduction (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). In *smo*$^{-}$ clones, Hh signalling is abolished (Chen and Struhl, 1996). Accordingly, *smo*$^{-}$ clones in A10-11 that abut A9 eliminated both Ptc (Fig. 4C) and En expression (Fig. 4D), indicating that En in the A10-11 was induced by Hh as occurs in the wing imaginal disc (Blair, 1992).

These results show that cells located anteriorly and posteriorly to the Hh source respond differently. While anterior A9 cells respond to Hh by activating Wg and/or Dpp, anterior A10-11 cells respond by activating En expression. The fact that Hh signals both infront and behind leads us to the issue of whether the segmental borders behave as compartment borders with respect to cell lineage restriction.

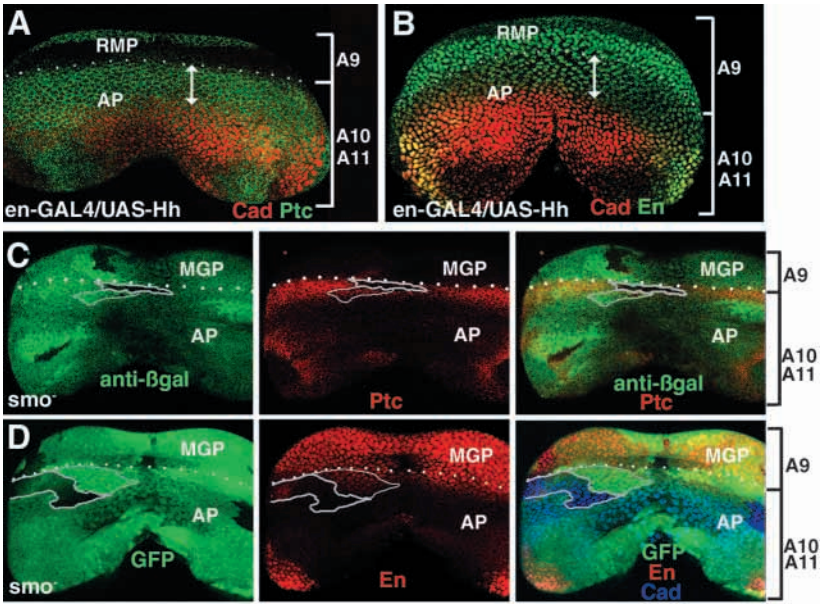

Fig. 4. Hh signal from posterior A9 induces En and Ptc expression in the most anterior part of A10-11. (A,B) Overexpression of Hh in the En domain of A9 using *en-Gal4/UAS-hh* expands (double arrow) Ptc (green, A) and En (green, B) expression and causes a reduction (double arrow) in Cad (red) expression in the most anterior part of A10-11. (C,D) *smo*$^{-}$ clones (which do not receive the Hh signal) in the most anterior part of A10-11 segment fail to express either Ptc (C) or En (D). A10-11 is labelled by the expression of Cad (blue). The *smo*$^{-}$ clones (thicker outline) and associated twin cells (thinner outline) are marked by β-gal (C) or GFP (D) expression (green).

Cell lineage restriction among genital disc primordia

Cell lineage restriction borders are formed at the interface between En/Hh and non-En/Hh expressing cells (reviewed by Dahmann and Basler, 1999). Hence, we tried to precisely determine the domains of En expression in the genital disc. This is not straightforward because of the complex folding of the disc. Carefully observation of En and Hh expression in male and female genital discs showed the three P compartments to be contiguous at the lateral edges of the disc. This was best observed in the A9 and A10-11 segments (Fig. 3A). Note that En expression is continued over the male genital primordium and the anal primordium (schematised in Fig. 1).

In the light of these observations, we re-analysed the behaviour of lineage clones. We first set out to determine whether the border between male and female genitalia constitutes a cell lineage restriction border. Secondly, we wanted to determine if Hh signalling, as in compartment borders, establishes the segmental borders.

To address the first question, we performed a clonal analysis in *dsx*^{1}/*dsx*^{1} genital discs, where both genital primordia grow at a similar rate and produce intersexual adult structures (P. C. Ehrensperger, PhD thesis, University of Zürich, Switzerland, 1983). Dübendorfer and Nöthiger (Dübendorfer and Nöthiger, 1982) have previously reported the existence of two separate cell lineages for the genitalia and analia in the developing genital discs. However, no cell lineage restriction between the male and female genitalia could be determined, as these authors were unable to detect any adult structures developing from the 'repressed primordia'. We induced β-gal clones in this mutant combination, between 0 and 24 hours of development, and monitored the clones in third instar larval genital discs. To distinguish the segmental border between male and female genital primordia, we took advantage of the specific expression of *teashirt* (*tsh*) in the A8 segment, thus labelling the female genital primordium (Fig. 5A). We observed that β-gal clones remained confined to either the male or female genital primordia, indicating the existence of a cell lineage restriction

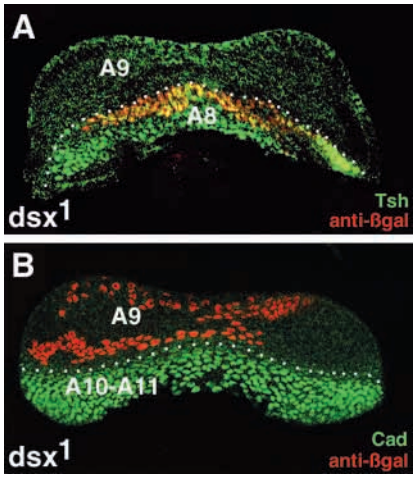

Fig. 5. Cell lineage restriction in *dsx*^{1} intersexual genital discs. (A,B) β-Gal-expressing clones (red) in *dsx*^{1} intersexual genital discs induced at 0-24 hours of development. (A) A β-gal-expressing clone in the A8 segment that does not cross to the A9 segment. To distinguish the segmental border between male and female genital primordia, we used an antibody against Teashirt (green) that is specifically expressed in the A8 abdominal segment (female genital primordium). (B) Clones in A9 do not cross to A10-11. We used the anti-Cad antibody (green) to distinguish the segmental border between the male primordium and the anal primordium.

barrier between both primordia (Fig. 5A). Similarly, β-gal clones in the male genital primordium did not cross towards the anal primordium (Fig. 5B).

It has been shown that at the AP compartment border of wing imaginal discs, A cells that lack *smo* and abut the AP border change their affinity and no longer mix with surrounding anterior cells but instead integrate with P cells. These cells were identified as clones of cells in the P compartment lacking Hh expression (thus of anterior identity) (Blair and Ralston, 1997; Rodríguez and Basler, 1997). To determine if the cell lineage restriction properties at the segmental border also depend on Hh signalling, we analysed the behaviour of *smo*$^{-}$ clones in the area around the border between A9 and A10-11 segments. Fig. 6A shows that *smo*$^{-}$ clones in the anterior compartment of A10-11 that abut the compartment border behave as described, i.e. they easily integrate among the P cells of the corresponding segment (detected as a clone of cells showing no *hh-lacZ* expression), while the twin clone remains at the A compartment (Fig. 6A, clone 3). However, *smo*$^{-}$ clones in the anterior compartment of A10-11 that abut the segmental border did not integrate with P cells of the A9 (male genital primordium). Instead, both the clone and the twin remained in the anterior A10-11 (Fig. 6B, clone 4). This result indicates that, besides Hh, some other factor was required to maintain the differential cell affinities at the segmental borders. Altogether, these findings suggest the segmental border does not behave as a compartment border, where the lineage properties of the cells are mainly dependent on Hh function. Instead, it seems that the segmental border between A9 (male genital primordium) and A10-11 (anal primordium) also depends on factors other than Hh and En.

The Hox genes are factors that might be involved in defining cell lineage restriction properties. These genes have segmental expression boundaries in the genital disc. Two Hox genes [*Abdominal B (Abd-Bm* and *Abd-Br*)] and *cad* are specifically expressed in the genital disc: *Abd-Bm* in A8, *Abd-Br* in A9 and *cad* in the A10-11 segments. It has been shown that *Abd B-m* and *r* specify the female and male genitalia (Estrada and Sánchez-Herrero, 2001) and *cad* specifies the analia (Moreno and Morata, 1999), and that *cad* lack of function clones autonomously activate *Abd-B* in the analia (Moreno and Morata, 1999). We thus explored the possibility that double mutant clones for *smo* and *cad* might be able to straddle the boundary between A9 and A10-11. *smo* and *cad* double mutant clones were incapable of straddling the segmental border between A9 and A10-11 (Fig. 6C, clones 5 and 6), suggesting that this segmental border is not dependent on the Hox genes.

Diffusion of morphogenetic signals across segmental borders

To determine whether there is a segmental restriction to the diffusion of morphogenetic signals in the genital disc, we ectopically expressed Hh in the analia, and examined its effect in the genitalia. *cad*-GAL4/UAS-*hh* females and males showed the complete duplication of external genital structures (Fig. 7C,D) and accordingly, duplication of the genital discs (Fig. 7E,F). These genital duplications were not produced when we autonomously activated the Hh pathway in the analia with a dominant negative form of *ptc (ptc*^{SSD}*)* (Martín et al., 2001) (data not shown). These results indicate that Hh from the analia is able to specify positional information in the more anterior segments, that is, in the genitalia. Spread of the Hh signal can occur through the common P compartments, as *en*-GAL4/UAS-*hh* also induced the same type of genital

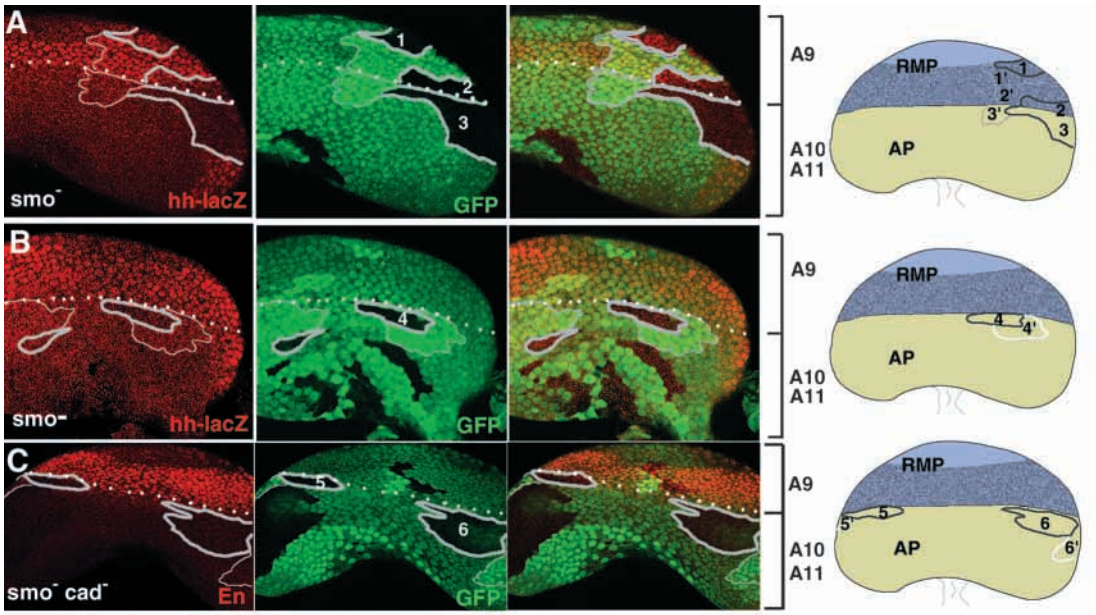

Fig. 6. The cell-lineage restriction border between segments A9 and A10-11. (A,B) Several samples of *smo*$^{-}$ clones induced in the region around the segmental border between segments A9 and A10-11. Clones (thinner outline) and their sister twins (thicker outline) are marked by GFP expression. The segmental border is marked by *hh-lacZ* expression. Clones 1 and 2 (A) are induced in the P compartment of A9, and clones 3 (A) and 4 (B) are located in the anterior compartment of A10-11. (C) *smo cad* double mutant clones (5 and 6) induced in the anterior region of A10-11. Note that 3, 4, 5 and 6 clones abut but do not straddle the segmental border between A9 and A10-11. See schemes on the right of the figure to locate the clones in the genital discs. 1′-6′ indicate the corresponding twin clones.

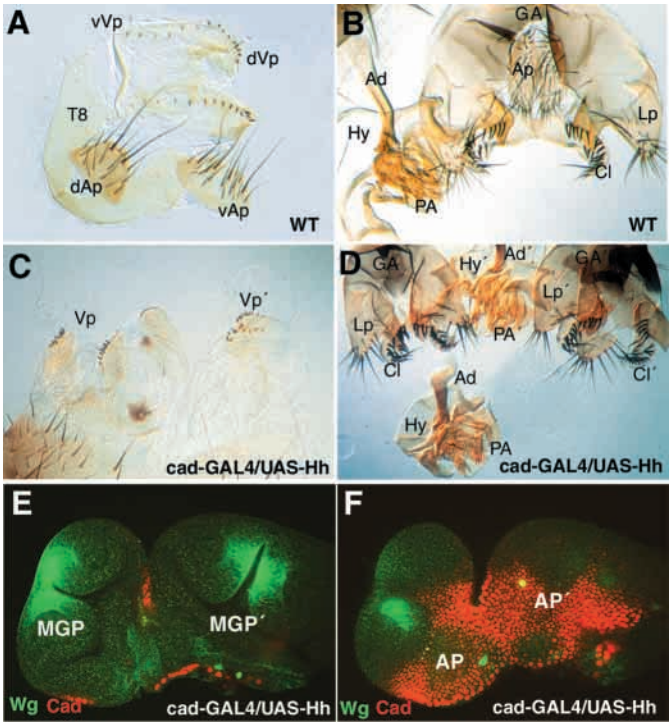

Fig. 7. Diffusion of Hh signal across segmental borders. (A-D) Ectopic Hh expression in the anal primordium in the *cad-Gal4/UAS-hh* combination duplicates genital structures both in females and males. (A,B) Wild-type structures of the female and male external terminalia. (C,D) Duplicated male genital disc *UAS-hh/cad-Gal-4*. (E,F) Duplicated male genital discs stained with anti-Wg (green) and anti-Cad (red). Ventral section (E) and dorsal section (F) of the same disc. vVp, ventral vaginal plate; dVp, dorsal vaginal plate; dAp, dorsal anal plate; vAp, ventral anal plate; T8, tergite eight; GA, genital arch; Ap, anal plate; Ad, apodeme; Lp, lateral plate; Cl, clasper; PA, penis apparatus; Hy, hypandrium; Vp′, vaginal plates; Ad′, Hy′; Vp′; PA′ are the duplicated structures. MGP, male genital primordium.

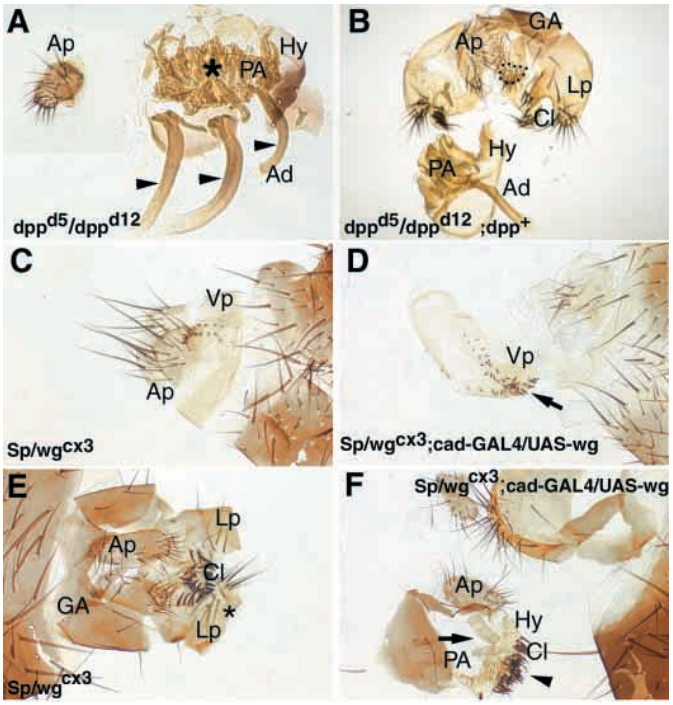

Fig. 8. Diffusion of Wg and Dpp signals across segmental borders. (A) The terminalia of dpp^{d5}/dpp^{d12} mutant males is very much reduced. Note the triplication of penis apparatus structures (asterisk) and the apodeme (arrowhead). (B) Ectopic *dpp* clones induced 24-48 hours after egg laying (indicated by a dotted line) non-autonomously recover the whole inventory of external genital structures, with the exception of one lateral lobe structure. (C) Phenotype of Sp/wg^{cx3} females with reduced vaginal plates and no vulva. In this mutant background, overexpression of Wg in the Cad domain (D) duplicates the vaginal plates (arrow). (E) Phenotype of Sp/wg^{cx3} males with no penis apparatus structures, and reduced claspers plus fused lateral plates (asterisk). Note the duplication of the lateral plate. In this mutant background, overexpression of Wg in the Cad domain (F) recovers the claspers (arrowhead) and some penis apparatus structures (arrow).

duplications (data not shown). We then tested whether the other morphogenetic signals, Wg and Dpp, were also able to diffuse across the segmental border between analia and genitalia.

Females of the mutant combination dpp^{d5}/dpp^{d12} had normal vaginal plates and tergite eight, showing only discretely reduced analia in a few cases. On the contrary, males displayed extensively reduced external terminalia (Fig. 8A), only presenting structures of the penis apparatus that could be duplicated or even triplicated. The development of the penis apparatus depends mainly on Wg (Sánchez et al., 1997), and due to the mutual repression between *wg* and *dpp*, Wg is expanded in a *dpp* mutant background, resulting in the duplications of the penis apparatus observed. To test the ability of Dpp to diffuse across the segmental border, we induced ectopic Dpp clones in a dpp^{d5}/dpp^{d12} mutant background. These clones always led to the recovery of the terminal structures (Fig. 8B). This recovery depended on the developmental stage at which the clone was induced. Thus, the earlier the clone was induced, the greater the inventory of structures differentiated by the genital disc. Recovery was accompanied by reduced penis apparatus structure duplications, and could be associated with duplications that mainly comprised the genital arch or lateral plate, two structures that require *dpp* activity (Sánchez et al., 1997). A property of all clones was their non-autonomous character: the Dpp-expressing clones marked with *yellow* always induced the development of unmarked structures. In some cases, the *yellow* bristles were only found in the analia (Fig. 8B), whereas in others they were found in the genitalia (data not shown). In either case, the corresponding genitalia and analia were also recovered. This non-autonomous effect of Dpp indicates that there is signal diffusion between the genital and the anal primordia, despite the fact that they belong to different segments.

To further test the ability of Dpp to spread through the genital-anal border, we specifically expressed Dpp in the analia in *cad-GAL4/UAS-dpp* flies and monitored effects in the genitalia. Complete duplication of the vaginal plates was observed (data not shown), in agreement with the effect induced by ectopic Hh. However, in some cases, all the terminalia were deleted (data not shown). Next, we tried to establish if the ability of ectopic Dpp in the analia to duplicate genital structures was due to Dpp itself or to downstream

signals activated by Dpp. We therefore tested the effect of the ectopic expression of a constitutive active form of a Dpp receptor, *thickvein* (*tkv*). The expression of this gene autonomously activates the Dpp pathway (Lecuit et al., 1996; Nellen et al., 1996). We found that *cad-GAL4/UAS-tkv* flies always lacked both analia and genitalia (data not shown). This indicates that the duplications obtained were not caused by a secondary signal activated by Dpp. Taken together these results indicate that Dpp is able to spread between the analia and genitalia.

Females flies of the *wg* mutant combination wg^{CX3}/Sp (*Sp* is also a *wg* allele) (Neumann and Cohen, 1996) have reduced vaginal plates and tergite eight, and the vulva is mainly absent (Fig. 8C). Mutant males have a strong phenotype in the genitalia: the penis apparatus and most of the clasper are absent and the lateral plates are fused (Fig. 8E). To analyse further the role of Wg in genital disc development as well as the non-autonomous effect of Wg, we overexpressed *wg* in the anal primordium in *cad-GAL4/UAS-wg*; wg^{CX3}/Sp flies. Some of the females showed recovery of the vaginal plates and tergite eight but not the vulva. The analia were much reduced in some cases (Fig. 8D). Other females showed no genital and anal structures. Males showed recovery of genital structures such as claspers and penis apparatus, although this latter structure was sometimes still reduced (Fig. 8F). Analia were almost normal or reduced. Thus, the ectopic expression of Wg in the analia can either recover structures of the genitalia or can prevent the development of both genitalia and analia.

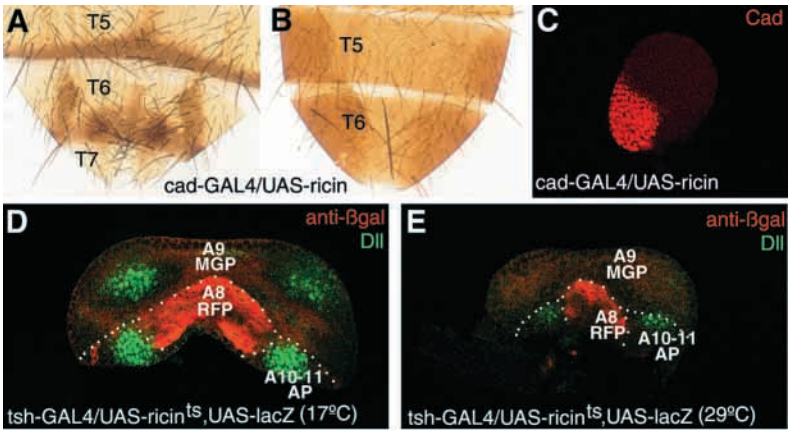

Fig. 9. Effect of ablation of the genital and anal primordium cells on the development of the genital disc. (A,B) Lack of terminal structures of female (A) and male (B) *cad-GAL4; UAS-ricin, UAS-lacZ* adults. (C) A very reduced *cad-GAL4; UAS-ricin, UAS-lacZ* genital disc. The anal primordium is labelled with anti-Cad antibody (in red). (D,E) Male genital discs expressing the cold-sensitive Ricin mutant protein in A8 (repressed female genital primoridum) using the *tsh-GAL4* line at the restrictive temperature (D) where the Ricin protein is not functional, and (E) at the permissive temperature. The discs are stained with anti-β-gal (in red) to label the A8 segment and with anti-Distal-less (in green) antibodies. Note the small size of the genital disc expressing functional Ricin protein in A8 and the lack of Distal-less expression in A9 (male genital primordium). Broken lines separate the three segmental primordia of the genital disc. T5, tergite 5; T6, tergite 6; T7, tergite 7.

To further explore the ability of Wg to spread through the genital-anal border, we specifically expressed Wg in the analia in *cad-GAL4/UAS-wg* flies, and monitored the effect on the genitalia. Small duplications of the penis apparatus or the complete absence of the terminalia were observed. We then overexpressed the downstream components of the Wg pathway Tcf (van de Wetering et al., 1997; Brunner et al., 1997; Riese et al., 1997) and a form of Armadillo (Arm) that is constitutively active (Zecca et al., 1996; van de Wetering et al., 1997). In both cases, the phenotype observed was the same as when Wg was ectopically expressed. Interestingly, diffusion of ectopic Wg protein from the analia towards the genitalia was not observed and the excess of Wg protein was confined to the analia of *cad-GAL4/UAS-*wg^{ts} genital discs (data not shown). This is in agreement with the observation that *en*-expressing cells make a barrier to the diffusion of Wg in the embryonic segment (Dubois et al., 2001) (see Discussion). These results suggest that a signal activated by Wg is responsible for the non-autonomous effect that the expression of this gene in the analia has in the genitalia.

In the experiments described above, we sometimes found that high level of Wg and Dpp in the analia impeded the development of both analia and genitalia. This suggested that the analia might be required for the development of the whole genital disc. To test this possibility, we induced ablation of the anal primordium cells using the toxic *UAS-ricin A* transgene that it has been shown to be efficient, cell specific and cell autonomous (Hidalgo et al., 1995; Hidalgo and Brand, 1997). *Ricin* A is the catalytic subunit of *Ricin* toxin, which is capable of killing a cell but not of crossing the cell membrane into neighbouring cells. In the form employed for transgenic expression in flies, *Ricin* lacks the subunit B and a secretory signal peptide, necessary for toxin internalisation and spreading to neighbouring cells (Moffat et al., 1992). *Ricin* A chain kills cells by depurinating 28S ribosomal RNA and effectively halting protein synthesis. We have observed that *cad-GAL4/UAS-ricin A* flies lack all the terminal structures (Fig. 9A,B). Dissection of these larvae at late third instar showed that the genital discs had not proliferated (Fig. 9C).

Next, to see the effect of ablation of cells of the genital primordia in the development of the genital disc, we expressed Ricin protein in the repressed female genital primordium (A8) of male genital discs by using *tsh-GAL4*. As *tsh* is expressed up to A9 segment during embryonic development, to avoid lethality of the embryos and allow them to develop into third larval stage so that the genital disc could be analysed, a cold-sensitive Ricin protein was used (Moffat et al., 1992). It was observed that inducing the functional Ricin protein at the second larval instar causes a reduction of proliferation of the genital disc (Fig. 9E) compared with a genital disc where the Ricin protein was non-functional during whole development (Fig. 9D). Note also that Distal-less expression was absent in the male genital primordium (A9) of the male genital disc that showed reduced proliferation (Fig. 9E).

Collectively, these results suggest that cell communication between the different primordia that form the genital disc is required for the development of all terminalia.

DISCUSSION

In the work presented, we examined the consequences of the genital disc being a complex disc formed by the fusion of three different segmental primordia, each composed of an anterior and a posterior compartment. We focused on two different, although related aspects: signal diffusion and cell lineage restriction between segmental primordia.

Interactions among segmental primordia are required for genital disc development

The three segmental primordia of the genital disc are contiguous. This means that the P compartment of one primordium is adjacent to A cells of the corresponding primordium and to A cells of the following primordium. In addition, the P compartment cells of the three segments converge in lateral areas of the genital disc (Keisman and Baker, 2001) (this paper). Hh is expressed in the three P compartments (Freeland and Kuhn, 1996; Casares et al., 1997; Chen and Baker, 1997). It is shown here, that Hh activates target genes in the receiving cells both behind and infront. These target genes are different on each side of its expression domain. Particularly, Hh at the posterior compartment of the male genital primordium (A9 segment) signals anteriorly, inducing Wg and/or Dpp expression in anterior cells of this primordium, and posteriorly, inducing Ptc expression. Hh also posteriorly signals anterior cells of the anal primordium (segments A10-11) inducing En expression in a narrow band of cells. Interestingly, Cad expression is reduced in these cells. A similar situation has been described in embryonic segments in which Hh activates *wg* at the AP border and *rhomboid* at the segmental border (O'Keefe et al., 1997; Alexandre et al., 1999). Hh controls Wg and EGF signalling pathways on each side of its expression domain in embryos. We therefore expected to find that, at the segmental border, Hh would activate a target gene implicated in a signalling mechanism. We tested specific expression at the segmental border of members of several signalling pathways, but unfortunately no such expression pattern was observed.

Hh has a pivotal role in the morphogenesis of all imaginal discs. Ectopic Hh gives rise to duplications of parts of, or whole, appendages in the imaginal discs of the fly (reviewed by Lawrence and Struhl, 1996). In wild-type genital discs, such as in the leg and antenna, Hh induces the expression of *wg* and *dpp* in A compartment cells close to the AP border of each of the three primordia. It is shown here, that the ectopic expression of *hh* in the anal primordium induces complete duplication of the genital disc with the corresponding expression of these genes in their normal expression domains. The repressed male and female primordia also seemed to be duplicated in the female and male genital discs, respectively. These results indicate again that Hh diffuses across the border between the genitalia and analia, although this border acts as a cell lineage restriction barrier (see below). It should be noted that Hh also diffuses across the border between the embryonic segments (Heemskerk and DiNardo, 1994) and the abdominal segments of adult flies (Struhl et al., 1997a; Struhl et al., 1997b; Lawrence et al., 1999). The results presented here also show that the ectopic expression of either *dpp* or *wg* in the analia also affects the development of the male and female genitalia.

The non-autonomous effect that ectopic Dpp in the analia has on the development of the genitalia is due to diffusion of Dpp itself from the analia to the genitalia, and not to the non-autonomous effect of Dpp downstream genes. By contrast, the same effect on the development of the genitalia was observed when Wg itself or any of the downstream components of the Wg-pathway, Tcf or Arm, were ectopically expressed in the analia. These results together with the observation that no Wg protein was detected in the genitalia when it was ectopically expressed in the analia indicate that the non-autonomous effect of ectopic Wg is due to an unknown signal activated by the Wg-pathway.

It has been recently described that Wg spreads and acts within the embryonic epidermis of *Drosophila* in different ranges in anterior and posterior directions (Sanson et al., 1999; Sanson, 2001; Dubois et al., 2001). Transport or stability is reduced in *engrailed*-expressing cells, and further posterior Wg movement is blocked at the presumptive segmental boundary. *hh* function is involved in the formation of this barrier. If Wg diffusion across the genitalia-analia border is limited, it might be established very early in development by a similar mechanism to that observed in the embryo.

The lack of development of the analia gives rise to a lack of genital structures, consistent with the outcome of genetic ablation of the analia by Ricin A (see above). This result indicates that morphogenetic signals diffuse from the analia to the genitalia, and that this diffusion is needed for the normal development of the genital disc. However, does diffusion also occur between the male and female genital primordia of the genital disc? The results obtained in the clonal analysis of *transformer* (*tra*) in the female genital disc are relevant to this question (Wieschaus and Nöthiger, 1982). It was found that male *tra*$^{-}$ clones could give rise to male genital structures associated with a loss of female genital structures (vaginal plates and tergite eight). These data suggest a communication system between both genital primordia. Results presented here support this contention. Ablation of cells of the repressed female genital primordium of a male genital disc by Ricin protein causes a reduction of proliferation of the genital disc.

In summary, the development of the genital disc, as that of other imaginal discs, requires interaction among the compartments forming each of its three primordia. The results presented here indicate that cell communication among different segmental primordia is also required for the development of the genital disc.

Cell lineage among genital disc primordia

A study of cell lineage in the male and female genital discs revealed that there is a cell lineage restriction between the analia and the female or the male genitalia (Dübendorfer and Nöthiger, 1982). No cell lineage restriction between the male and female genitalia could be determined as each of these does not develop in the opposite sex and consequently does not produce adult structures. For this reason, we performed a clonal analysis in intersexual flies in which both genital primordia develop. We found that there is a cell lineage restriction barrier between female (A8) and male (A9) primordia, and between male and anal (A10-11) primordia. Even though the P compartment cells of the three segments converge in the lateral areas of the disc, there is still a cell lineage restriction among the P cells of different segments. In other imaginal discs, such as those of the wing and leg discs –

composed of an A and P compartment – the cell lineage restriction between A and P cells is a consequence of the different affinity of posterior En/Hh expressing cells and anterior non-En/Hh expressing cells.

The present results indicate that Hh signalling is not responsible for the cell lineage restriction between segmental primordia between A9 and A10-11. The question arises as to how this cell lineage restriction is achieved. We found that the Hox gene *cad*, which is expressed only in the anal primordium (Moreno and Morata, 1999), is not required for the restriction at the segmental border between the male genital primordium (A9) and anal primordium (A10-11). The gene *Abd-B*, which produces two different Abd-Bm and Abd-Br proteins (Casanova et al., 1986; DeLorenzi et al., 1988; Kuziora and McGinnis, 1988; Sánchez-Herrero and Crosby, 1988), is expressed in the genital primordia. Abd-Bm is only present in the female genital primordium (A8), whereas Abd-Br is only found in the male genital primordium (A9) (Casares et al., 1997; Estrada and Sánchez-Herrero, 2001). This suggests that the Hox genes might not only have a role in defining the identity of each segment of the genital disc (Estrada and Sánchez-Herrero, 2001) but may also be involved in establishing the cell lineage restriction among segmental boundaries. However, we found that *smo; cad* double mutant clones were still unable to straddle the segmental boundary between the A9 and A10-11 segments. Moreover, we observed the overlapping of Abd-B and Cad expression domains at the border between A9 and A10-A11 (data not shown). Thus, this segmental boundary does not behave like other segmental boundaries where the Hox expression domains are exclusive. Nevertheless, it is still possible that the interface between Abd-Bm and Abd-Br expressing cells forms the A8-A9 lineage restriction barrier.

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