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The *Drosophila* sex determination hierarchy modulates *wingless* and *decapentaplegic* signaling to deploy *dachshund* sex-specifically in the genital imaginal disc

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SUMMARY

The integration of multiple developmental cues is crucial to the combinatorial strategies for cell specification that underlie metazoan development. In the *Drosophila* genital imaginal disc, which gives rise to the sexually dimorphic genitalia and analia, sexual identity must be integrated with positional cues, in order to direct the appropriate sexually dimorphic developmental program. Sex determination in *Drosophila* is controlled by a hierarchy of regulatory genes. The last known gene in the somatic branch of this hierarchy is the transcription factor *doublesex* (*dsx*); however, targets of the hierarchy that play a role in sexually dimorphic development have remained elusive. We show that the gene *dachshund* (*dac*) is differentially expressed in the male and female genital discs, and plays sex-specific roles in the development of the genitalia. Furthermore, the sex determination hierarchy mediates this sex-specific deployment of *dac* by modulating the regulation of *dac* by the pattern formation genes *wingless* (*wg*) and *decapentaplegic* (*dpp*). We find that the sex determination pathway acts cell-autonomously to determine whether *dac* is activated by *wg* signaling, as in females, or by *dpp* signaling, as in males.

Key words: *Drosophila*, Genital disc, Sex determination, *wingless*, *decapentaplegic*, Pattern formation

INTRODUCTION

A variety of molecular and genetic studies have revealed that many important regulatory molecules are used to control diverse developmental processes. For example, signal transduction pathways such as the receptor tyrosine kinase/Ras/mitogen-activated protein kinase pathway (Tan and Kim, 1999), cell-cell signaling molecules such as Notch and its ligands (Artavanis-Tsakonas et al., 1999), and morphogens such as Wingless/WNT (Cadigan and Nusse, 1997) and Dpp/TGFβ (Massague and Wotton, 2000) are deployed during the differentiation of multiple cell and tissue types, with context-specific outcomes. This presents one of the major outstanding questions in developmental biology: how is the context-specific interpretation of generic developmental signals achieved?

Sexual differentiation in *Drosophila melanogaster* is an attractive system in which to address this question. It involves the integration of a simple, binary fate choice (male or female) with a host of developmental decisions in dimorphic tissues that range from leg bristles to the central nervous system (Cline and Meyer, 1996; Ryner et al., 1996). The most extensive sexual dimorphism is found in the male and female genitalia, both of which are derived from the genital imaginal disc (reviewed by Lauge, 1982). The genital disc is a compound disc that comprises the primordia of the female genital, male genital and anal structures. In females, the female genital primordium develops, while the male genital primordium is repressed and the anal primordium takes on the female form. Conversely, in males, the female primordium is repressed, the male primordium develops, and the anal primordium takes on the male form. Thus, by late third instar, the genital disc exhibits a characteristic sexual dimorphism, with the active and repressed genital primordia occupying stereotypical places within the epithelium (Fig. 1A,B).

Somatic sex determination outside of the central nervous system in *Drosophila* is controlled by a well characterized hierarchy of regulatory genes (Fig. 2), whose function culminates in the production of sex-specific proteins encoded by the *doublesex* (*dsx*) locus (Cline and Meyer, 1996). The master regulatory gene *Sex lethal* (*Sxl*) is activated only in females and directs the female-specific splicing of the *transformer* (*tra*) pre-mRNA, so that an mRNA encoding Tra protein is produced. Together with the product of the *transformer-2* (*tra-2*; *tra2* – FlyBase) locus, Tra directs the female-specific splicing of the *dsx* pre-mRNA, producing an mRNA that encodes the Dsx^f protein. In males, where Sxl protein is absent, *tra* pre-mRNA is spliced by default in the male pattern, producing an mRNA that does not encode functional Tra protein. In the absence of Tra protein, *dsx* pre-

mRNA is spliced by default into the male-specific mRNA, which encodes the Dsx^m protein. *dsx* is the last gene in the somatic sex-determination hierarchy and controls all aspects of somatic sexual differentiation outside of the central nervous system.

The male- and female-specific Dsx proteins are transcription factors that share a common zinc-finger DNA-binding domain (Erdman and Burtis, 1993). The only genes that are known to be directly regulated by the Dsx proteins are the yolk protein genes *yp1* and *yp2* (*Yp1* and *Yp2* – FlyBase). The *yp* genes are expressed in the fat bodies of females, but not males. This restricted expression is the result of the coordinate regulation of both *yp* genes by Dsx and tissue-specific factors acting on a compact regulatory element, the fat body enhancer (Garabedian et al., 1986; Logan et al., 1989; Burtis et al., 1991; Coschigano and Wensink, 1993; An and Wensink, 1995a; An and Wensink, 1995b). Consistent with an instructive role for the *dsx* locus, both male and female Dsx proteins are required for proper *yp* gene regulation: Dsx^f activates transcription of the *yp* genes in females and Dsx^m represses their transcription in males (Coschigano and Wensink, 1993). Evidence suggests that regulation by *dsx* is superimposed on tissue-specific regulation by direct interaction of Dsx proteins with tissue-specific transcription factors (An and Wensink, 1995a; An and Wensink, 1995b).

One unanswered question with respect to genital disc development concerns whether *dsx* plays a permissive or an instructive role in the differentiation of the genitalia. In the absence of *dsx* function, both male and female genitalia differentiate (Baker and Ridge, 1980), although these genitalia are frequently incomplete. This result suggests that the primary role of the sex-specific Dsx proteins is simply to specify which genital primordium will develop. In this 'permissive' model, the male or female Dsx protein represses the inappropriate genital primordium; other selector genes would then specify which structures differentiate from the primordium that does develop. However, several lines of evidence suggest that *dsx* plays an instructive role in sexual differentiation. In particular, there is evidence that the male and female primordia are somewhat plastic, and can give rise to elements usually restricted to the opposite sex. Thus, certain *dsx* mutants, in addition to a more or less fully developed male genitalia, differentiate an extra, rudimentary set of male genitalia. These extra male structures are frequently found intermingled with the female genitalia, suggesting that they derive from the female genital primordium (Baker and Ridge, 1980; Epper, 1981). This phenotype is also produced by temperature sensitive alleles of *tra-2* when a developing female primordium is shifted to the male-determining temperature late in development (Belote and Baker, 1982; Sanchez and Granadino, 1992). Late shifts from the male- to the female-determining temperature hinted that the male primordium has an analogous capacity to differentiate rudimentary female structures, though this result was not conclusive (Belote and Baker, 1982). Insight into the role of *dsx* in genital disc development might be gained by the discovery of genes whose expression in the genital primordia is regulated by *dsx*.

One approach to identifying potential *dsx* targets in the genital disc is to look for genes that are expressed in sex-specific patterns in that tissue. We reasoned that such targets might be coordinately regulated by *dsx* and the genes that control pattern formation in imaginal discs. Therefore, as a prelude to this effort, we characterized the role of known pattern formation genes in the genital disc (Chen and Baker, 1997). We and others have shown that the same genetic hierarchies that control pattern formation in the thoracic imaginal discs function analogously in the genital disc (Freeland and Kuhn, 1996; Chen and Baker, 1997; Casares et al., 1997; Sanchez et al., 1997; Emerald and Roy, 1998). Within each of the three genital primordia, there is an anterior and a posterior compartment, specified by the *engrailed* (*en*) gene. As in the leg disc, *en* acts through the secreted protein encoded by *hedgehog* (*hh*) to de-repress *wingless* (*wg*) and *decapentaplegic* (*dpp*) in complementary and mutually exclusive domains along the anterior/posterior (A/P) border. *wg* and *dpp*, in turn, encode secreted morphogens that specify positional information (Gelbart, 1989; Klingensmith and Nusse, 1994; Lecuit et al., 1996; Nellen et al., 1996). While the paired leg discs each possess a dorsal stripe of *dpp* expression and a ventral stripe of *wg* expression, the bilaterally symmetric, unpaired genital disc has a single, medial *wg* expression domain that is flanked by two lateral *dpp* expression domains. The mutually exclusive domains of *wg* and *dpp* expression are maintained by a system of mutual antagonism: in the leg disc, for example, ventral *wg* expression represses *dpp* expression to confine it dorsally; *dpp*, in turn, represses *wg* expression to confine it ventrally (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996).

While studying pattern formation in the genital disc, we discovered that the *dachshund* (*dac*) gene, a known target of *wg* and *dpp* regulation in the leg disc, is expressed sex specifically in the genital disc. In the leg disc, *dac*, which is required for the differentiation of mid-proximal structures, is expressed in a mid-proximal ring (Mardon et al., 1994). To produce this expression pattern, intermediate levels of the Wg and Dpp proteins co-operate to activate *dac* in the mid-proximal leg, while high levels of Wg and Dpp in turn repress *dac*, excluding *dac* expression from the distal leg (Lecuit and Cohen, 1997). Other genes such as *homothorax* refine this regulation in order to bring about the final pattern of *dac* expression (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998; Goto and Hayashi, 1999; Wu and Cohen, 1999).

We report that in the male genital disc, *dac* is activated by *dpp* and repressed by *wg*, while in female genital discs, the converse relationship exists. This results in the sex-specific deployment of *dac* to different regions of the genital primordia, where we show it is required for the appropriate differentiation of both male and female genital structures. Furthermore, we demonstrate that the sex-determination pathway acts cell-autonomously to modulate the regulatory relationship between *wg*, *dpp* and *dac*. This finding constitutes the first demonstration that the sex determination pathway plays an instructive role in the sex-specific differentiation of the genitalia.

MATERIALS AND METHODS

Stocks

The following mutant alleles and transgenes were used in this study: UAS-*wg* (H. Krause, unpublished); *UAS-tkv** (Nellen et

al., 1996); Actin>CD2>GAL4 (Pignoni and Zipursky, 1997); *Actin>y$^+$>GAL4,UAS-GFP* (Ito et al., 1997); *UAS-dpp 37A.2* (Staehling-Hampton and Hoffmann, 1994); *UAS-tra* (Ferveur et al., 1995); *UAS>arm** (Zecca et al., 1996); *wg-lacZ* and *dpp-lacZ* are inserts of a *lacZ* enhancer trap into the *wg* and *dpp* loci, respectively (Tabata and Kornberg, 1994); UAS-*tra2-IR* (Fortier and Belote, 2000); *UAS-GFP.S65T* is an insertion on the 2nd chromosome (http://flybase.bio.edu/.bin/fbpcq.html?FBrf0086268); *dpp^{d12}* and *dpp^{d14}* (Spencer et al., 1982; St Johnston et al., 1990); *wg^{CX3}* and *wg^{CX4}* (Baker, 1987); *dsx^{D+R3}* (Duncan and Kaufman, 1975); *dsx^{M+R15}* (Baker et al., 1991); *hsflp1* is an insertion on the first chromosome (Golic and Lindquist, 1989); *hsflp* on the MKRS balancer chromosome (Chou and Perrimon, 1992); *hsflp122* is synonymous with *hsflp.1* (Jiang and Struhl, 1995).

hsflp122 is expressed at much higher levels than *hsflp1* under comparable heat shock conditions and is 'leaky', in that it can cause flip-out in the absence of heat shock when larvae are raised at 25°C (E. K., unpublished observations). Therefore, we only used it in one experiment, in which we wished to induce flip-out in virtually every single cell (see below).

Antibody staining

Tissues were prepared for antibody staining by fixation in fresh, ice-cold PLP (4% paraformaldehyde, 75 mM lysine, 11 mM $NaIO_4$) and were then fixed overnight at 4°C. Washes were carried out at 4°C in 0.1M $NaPO_4$ pH 7.2, 0.1% saponin. Samples were incubated in antibody diluted into 0.1M $NaPO_4$ pH 7.2, 0.1% saponin, 2% normal goat serum. Antibody dilutions were as follows: mouse anti-Dac 2-3 (a generous gift from Graeme Mardon) 1:200; Cy3 anti-mouse and Cy5 anti-Rabbit (Jackson Immunoresearch), 1:200; rabbit anti-β-gal (Cappel), 1:5000. The genital disc was stained while attached to inverted larval abdomens, then dissected and mounted in VectaShield (Vector Laboratories). *wg* and *dpp* expression patterns were detected by staining genital discs from larvae carrying *wg-lacZ* or *dpp-lacZ* reporter genes. Samples were observed using a Zeiss Axiophot fluorescent microscope, or imaged using a BioRad MRC1024 confocal microscope.

Staging of pupae

Pupae were collected as white pre-pupae and raised to the indicated time at 25°C.

Ectopic expression using the flip-out GAL4 system

GFP-marked clones that ectopically express the genes indicated below were created using the flip-out GAL4 system (Ito et al., 1997; Pignoni and Zipursky, 1997). This system is derived from a combination of the flip-out technique described by Struhl and Basler (Struhl and Basler, 1993) and the GAL4/UAS technique for ectopic gene expression described by Brand and Perrimon (Brand and Perrimon, 1993).

Clones that ectopically express *wg* were generated in larvae of the genotype *y w hsflp1/yw; UAS-wg/ Actin>y$^+$>GAL4, UAS-GFP*. Clones that ectopically express *arm** were generated in larvae of the genotype *y w hsflp1/yw; Actin>y$^+$>GAL4, UAS-GFP/+; UAS>arm*/+* or *y w hsflp122/yw; Actin>y$^+$> GAL4, UAS-GFP/+; UAS>arm*/+*. The latter genotype was used to induce flip-out *arm** in nearly every single cell in the genital disc; expression from the *hsflp122* transgene is so strong that a single 30 minutes heat shock at 37°C will accomplish this.

Clones that ectopically express dpp were generated in larvae of the following genotypes: y w hsflp1; UAS-dpp/Actin>y$^+$>GAL4, UAS-GFP (see Fig. 6A); Actin>CD2>GAL4; UAS-dpp, wg-lacZ/UAS-GFP; MKRS, hsFLP/+ (see Fig. 6B); Actin>CD2>GAL4/y w hslflp1; UAS-dpp, wg-lacZ/UAS-GFP; MKRS, hsFLP/+ (see Fig. 6E). Clones that ectopically express tkv* were generated in larvae of the genotype Actin>CD2>GAL4; UAS-GFP/+; UAS-tkv*/MKRS, hsFLP.

Clones that ectopically express the female *tra* cDNA (*tra$^+$* clones) were generated in larvae of the genotype *y w hsflp1/yw; UAS-tra/Actin>y$^+$>GAL4, UAS-GFP*. Larvae in which *tra$^+$* clones were induced were raised at 25°C and then shifted to 18°C after heat shock. This regimen was adopted when it was discovered that at 25°C, ubiquitous GAL4-driven *UAS-tra*, in addition to feminizing males, has a dominant negative effect that masculinizes females. At 18°C, GAL4 drives expression at lower levels; ubiquitous GAL4-driven *UAS-tra* under these conditions transforms XY males into grossly normal females without masculinizing XX females (E. K., unpublished observations).

Clones in which *tra-2* function was blocked by expression of the *tra-2* inverted repeat *UAS-tra2-IR* (*tra2IR* clones) were generated in larvae of the genotype *Actin>CD2>GAL4/w,UAS-tra-2IR; UAS-GFP/+; MKRS, hsFLP /UAS-tra-2-IR*.

Larvae in which *tra2IR* clones were induced were raised at 25°C and shifted to 29°C after heat shock to maximize GAL4-driven expression from the UAS promoter. These conditions create the strongest *tra2* loss-of-function phenotype (Fortier and Belote, 2000). Sibling males are of the genotype *Actin>CD2>GAL4/Y; UAS-GFP/+; MKRS,hsflp/UAS-tra-2-IR*, and thus have only one copy of the *UAS-tra2-IR* transgene. *tra2IR* clones had no effect on *dac* expression in the male genital disc, as expected (data not shown).

Heat-shock conditions varied as different genotypes and times of clone induction necessitated. For example, owing to the perdurance of *tra2* gene product (Baker and Ridge, 1980; Wieschaus and Nothiger, 1982) we wanted to induce *tra2IR* clones in the first instar. Therefore, a stronger heat shock was required, owing to the relative inefficiency of clone recovery when clones are induced at this time. Times and durations of heat shock were: 48-72 hours after egg laying (hAEL), 37°C × 30 minutes (see Fig. 5A); 24-48 hAEL, 37°C × 30

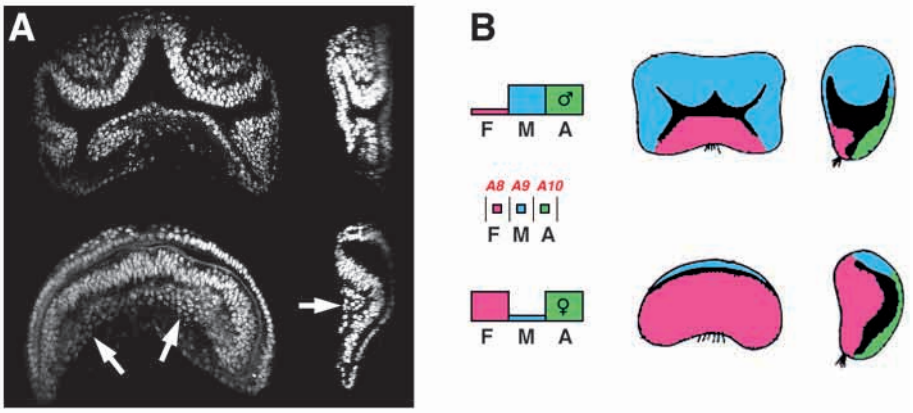

Fig. 1. The genital disc and its development. In all panels anterior is upwards; sagittal sections are oriented with ventral towards the left. (A) Confocal images and parasagittal reconstructions of the genital disc stained to reveal nuclei. Top, male genital disc; bottom, female genital disc. Note the mass of loosely packed adepithelial cells that clings to the female genital disc (arrows). (B) Origin and topology of the genital disc primordia. The genital disc primordia derive from segments A8 (female, F), A9 (male, M) and A10 (anal, A). In males (top), the female primordium is repressed, the male primordium grows, and the anal plates take on the male fate. In females (bottom), the male primordium is repressed, the female primordium develops, and the anal plates take on the female fate. Note the relative positioning of the primordia in the mature disc (adapted from F. Epper, PhD thesis, University of Zurich, 1980).

minutes (see Fig. 5B); 48-72 hAEL, 37°C × 30 minutes (see Fig. 5C-H); 48-72 hAEL, 37°C × 40 minutes (see Fig. 6A); 72-96 hAEL, 37°C × 45 minutes (see Fig. 6B-G); 24-48 hAEL, 37°C × 40 minutes (see Fig. 7A,B); 24-48 hAEL, 37°C × 45 minutes (see Fig. 7C,D).

Mounting of adult genitalia

Male genitalia were dissected in PBS, boiled for 5 minutes in 10% KOH to remove soft tissue, dehydrated in an ethanol series, equilibrated in acetone and mounted in Araldite for observation. Female internal and external genitalia were dissected in PBS, fixed in freshly made 3.7% formaldehyde in PBS overnight to preserve soft tissue, dehydrated in an ethanol series, equilibrated in acetone and mounted in Araldite for observation.

Statistics

Statistical analyses were performed using StatView (Abacus Concepts).

RESULTS

dac expression in the genital disc is sex specific

The genital imaginal disc is unique in structure and organization among the imaginal discs in *Drosophila*. Although the thoracic discs each derive from a single segment, the genital disc actually consists of three imaginal primordia: the female primordium, derived from abdominal segment A8, the male primordium (A9) and the anal primordium (A10). While the thoracic discs exist in pairs, the genital disc is unpaired and bilaterally symmetric. Finally, the development of the genital disc is sexually dimorphic, as only the appropriate genital primordium develops in each sex, while the inappropriate primordium is consigned to a 'repressed' state (Nothiger et al., 1977; Schupbach et al., 1978; Belote and Baker, 1982; Dubendorfer and Nothiger, 1982; Epper and Nothiger, 1982). These factors conspire to produce the morphology seen in the third instar genital discs (Fig. 1A,B): the female genital disc has a dorsal and a ventral epithelium. The highly columnar ventral epithelium consists of the female genital primordium; the dorsal epithelium is made up in the anterior by the repressed male primordium (RMP), with the posterior third comprising the anal primordium. In the male genital disc, the male primordium develops to produce a highly folded ventral epithelium and a thin dorsal epithelium. The anal primordium retains its position at the posterior of the dorsal epithelium. While the RMP is integrated seamlessly into the epithelium of the female disc, the repressed female primordium (RFP) is clearly set aside at the ventral posterior end of the male genital disc.

Most pattern formation genes, such as *en*, *patched*, *Distal-less* and *optomotor blind* (*bifid* – FlyBase) are expressed in domains that are homologous between the male and female disc, and that reflect the maintenance of the regulatory relationships between these genes that have been described for the leg disc (Chen and Baker, 1997; Sanchez et al., 1997; Gorfinkiel et al., 1999; E. K., unpublished observations). The expression patterns of *wg* and *dpp* are good examples: in both male and female genital discs, *wg* is expressed in a single medial domain along the A/P border (Fig. 3A,B) that is complementary to the two *dpp*-expressing domains that flank it laterally (Fig. 3C,D). In contrast, we found that *dac* expression is radically different between the male and female genital disc. In the female genital disc, *dac* is expressed in a medial domain centered around *wg* expression, while in the male genital disc, *dac* is expressed in two lateral domains that abut and partially overlap with *dpp* expression (Fig. 3E,F). On closer examination, the female *dac* domain reveals itself to be composed of a swathe of expression in the ventral female primordium and a broad domain in the anterior of the RMP, which also expresses *wg* in a thin band of cells. Each of the two male *dac*-expressing domains begins at the lateral edge of the ventral epithelium, wraps around this edge and spreads out onto the dorsal epithelium.

In order to understand the role of *dac* in the differentiation of the genitalia, we needed to know which adult structures were derived from the *dac*-expressing cells in the third instar genital disc. Owing to the complex metamorphosis of the genitalia, the correlation between imaginal disc expression patterns and adult structures is not as readily apparent in the genital disc as it is in thoracic discs, so we examined the expression pattern of *dac* during metamorphosis in wild-type animals. The expression of *dac* is shown in Fig. 4 at 48 hours after puparium formation (APF), when the fate of the *dac*-expressing cells is clear. In the female (Fig. 4A,C), the *dac*-expressing cells contribute to the ducts that connect the spermethecae and parovaria to the uterus, as well as to the region of the uterine wall from which these ducts originate. In the male (Fig. 4B,D), *dac* is expressed in what will become the clasper teeth. According to the fate maps of the male and female genital disc (P. Ehrensperger, Diplomarbeit, University of Zurich, 1972; Epper, 1983b), the structures that express dac at 48 hours APF are likely derived from the same population of cells that expresses dac in the third instar disc.

To ask whether *dac* is required in the adult structures whose precursors express *dac* at 48 hours APF, we examined the

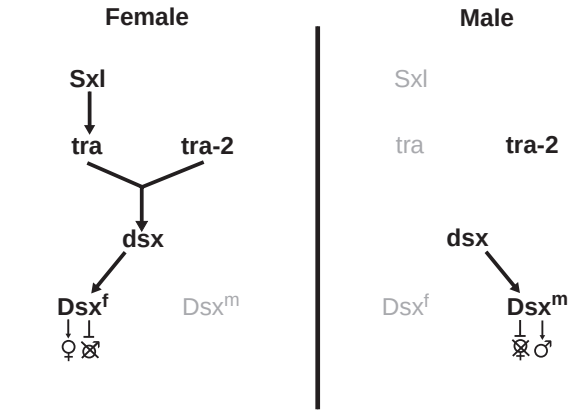

Fig. 2. The sex determination hierarchy. Shown is an abbreviated version of the somatic sex determination hierarchy (for a more complete description, see Meyer and Cline 1996). *Sxl* is activated only in females and triggers female differentiation. *Sxl* directs the productive splicing of *tra* pre-mRNA so that Tra protein is made. Tra, together with the product of the *tra-2* locus, directs the female-specific splicing of *dsx* pre-mRNA, so that it encodes Dsx^f. Dsx^f represses male differentiation and activates female differentiation. Males lack *Sxl* (gray), and thus lack functional *tra* product as well. Absent Tra, *dsx* pre-mRNA is spliced by default in the male-specific pattern which encodes Dsx^m. Dsx^m represses female differentiation and activates male differentiation.

genitalia of dac^3 pharate adults that were dissected from their pupa cases. dac^3 is a null allele, and dac^3 animals rarely eclose successfully, owing to their truncated legs. The female *dac* phenotype is quite subtle. In wild-type females, each of the two spermethecae has a duct that connects it to the uterus (Fig. 4C, E). These ducts attach to the uterus side by side. *dac* mutant females still have two spermethecae, but the two ducts are fused into one branched duct that is shared by both spermethecae (Fig. 4G). Compared with wild type, male *dac* null flies exhibit a severe reduction of the clasper (Fig. 4H). The claspers are truncated, have a reduced number of clasper teeth bristles, and lack the long bristle at their 'distal' end, as has been previously reported (Gorfinkiel et al., 1999). Thus, *dac* is deployed in a sex-specific fashion to non-homologous regions of the male and female genital discs, where it plays a role in the differentiation of adult genital structures.

wg activates *dac* in the female genital disc and represses *dac* in the male

The coincidence of *wg* and *dac* expression in the female genital disc, taken together with the knowledge that Wg activates *dac* in the leg discs (Lecuit and Cohen, 1997), strongly suggests that there is also a regulatory relationship between these two genes in the genital disc. Moreover, the absence of *dac* from the *wg*-expressing domain in the male genital disc suggests that this regulatory relationship is sex specific. To elucidate the regulatory relationship between *wg* and *dac*, we examined the affect of *wg* loss- and gain-of-function on *dac* expression in both male and female genital discs.

wg function was removed using a heteroallelic combination of the *wg* alleles wg^{CX3} and wg^{CX4}. This combination of alleles has been shown to provide sufficient *wg* function for embryonic development, but adults show loss of structures whose fates are specified by *wg*, presumably owing to impaired *wg* function in imaginal discs (Baker, 1987; Baker, 1988). Imaginal discs were dissected from wg^{CX3}/wg^{CX4} larvae and stained with an anti-Dac monoclonal antibody. Although these mutant discs are smaller than wild type, the effect on *dac* expression is unambiguous: in the female genital disc, *dac* expression is absent or severely reduced (Fig. 3H). Occasionally a small area of low-level *dac* expression remains (arrow, Fig. 3H). Male discs, on the other hand, show a dramatic expansion of *dac* expression, which now spans the entire disc (Fig. 3G). Thus, removing *wg* function not only reveals a sex-specific requirement for *wg* to activate *dac* in females, but shows a probable involvement for *wg* in restricting *dac* expression to the lateral domains in the male genital disc.

We wished to determine if *wg* expression was sufficient, as well as necessary, to activate *dac* expression. We assessed the effect of ectopically expressing *wg* on *dac* expression by using the flip-out GAL4 system (Ito et al., 1997; Pignoni and Zipursky, 1997) to create GFP-marked clones that express *wg* at novel, random locations in the genital disc. In the female genital disc, the effect of these clones on *dac* expression varies, depending on their location within the disc. *wg*-expressing clones in the dorsal RMP clearly cause ectopic *dac* expression both in and around the clone (Fig. 5A). Clones in the ventral, female primordium have a more ambiguous behavior. Ventral clones do not reliably cause ectopic *dac* expression; clones that do express *dac* do so at levels below that of the endogenous *dac* domain (Fig. 5B). A possible explanation for the ambiguous behavior of ventral *wg*-expressing clones comes from the fact that *dpp* is known to antagonize *wg* function. *dpp* is not expressed in the dorsal RMP, where *wg*-expressing clones more reliably activate *dac*, and the ventral regions where *dpp* is expressed in the female genital disc are near the regions where *wg* expression activates *dac* poorly (see Fig. 3D). Furthermore, we noticed that the few *dac*-activating clones that were found in the ventral primordium show a non-uniform

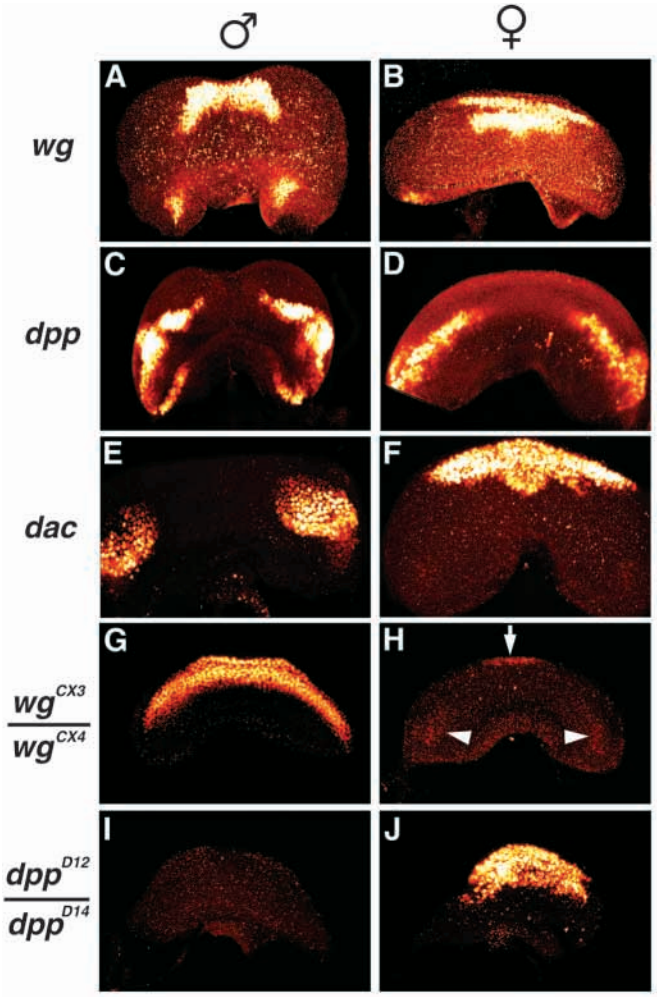

Fig. 3. *wg*, *dpp* and *dac* in the genital disc. (A-F) Confocal images of genital discs showing the expression patterns of *wg* (A,B), *dpp* (C,D) and *dac* (E,F) in the male (left) and female (right). Note that *dac* expression correlates roughly with that of *wg* in the female disc, but with that of *dpp* in the male genital disc. *wg* and *dpp* expression were detected using *lacZ* reporter lines stained with anti-β-gal antibodies. *dac* expression was revealed by staining with an anti-Dac monoclonal antibody. (G-J) *dac* expression in genital discs from mutant larvae. (G,H) Third instar genital discs from wg^{CX3}/wg^{CX4} mutant larvae. *dac* expression in the male genital disc (G) expands across the disc (7/7 discs), while in the female disc (H), *dac* expression is severely reduced (13/13 discs). Arrow and arrowheads indicate faint remnant *dac* expression. (I,J) Third instar genital discs from dpp^{d12}/dpp^{d14} mutant larvae. *dac* expression is virtually undetectable in the male disc (15/15 discs) (I) but remains in the female disc (16/16 discs) (J), even though the female disc is severely reduced. The images in H,I have been artificially brightened so that the outlines of the discs can be seen.

distribution of *dac* expression within the clone: ectopic *dac* expression is restricted to the regions of these clones that are furthest from the *dpp*-expressing domain (Fig. 5B, inset).

We reasoned that if *dpp* prevents *wg*-mediated activation of *dac* in the female primordium, we might overcome the effect of *dpp* by constitutively activating the *wg* signal transduction pathway. *armadillo* (*arm*) is the most downstream effector of the *wg* signal transduction pathway (reviewed in Dierick and Bejsovec, 1999); a modified version of the *arm* gene product that lacks a negative regulatory domain, *arm**, is known to be constitutively active (Zecca et al., 1996; Pai et al., 1997). We used the flip-out GAL4 system to make GFP-marked clones in the genital disc that express *arm**. These *arm**-expressing clones recapitulate the results obtained using ectopic *wg* expression (data not shown). We observe sex-specific activation of *dac*, but this is still restricted to specific regions of the female disc. It was difficult to tell with certainty where *arm** clones could and could not activate *dac*, owing to their tendency to sort from the surrounding epithelium. Ubiquitous activation of *wg* signaling would be expected to prevent the expression of *dpp* in the ventral primordium, perhaps allowing the *arm**-expressing cells to activate *dac*. Therefore, in a second experiment, we used strong heat-shock conditions that cause *arm** to be expressed in nearly every cell in the genital disc (see Materials and Methods). In the female genital disc, near-ubiquitous expression of *arm** causes *dac* to be activated ectopically in the ventral primordium (Fig. 5C,D). These results suggest that the failure of many *wg*- and *arm**-expressing clones to activate *dac* in the ventral primordium of the female disc in earlier experiments is indeed due to the ability of *dpp* to repress *dac* there. Consistent with this interpretation, occasional wild-type patches of cells cause non-autonomous repression of *dac* in the *arm**-expressing cells around them (Fig. 5D, arrows). A wild-type patch of cells in the presumptive *dpp*-expressing domain would allow *dpp* expression; Dpp could then spread into the surrounding *arm** cells and prevent them from expressing *dac*. It is not clear whether *dpp* acts indirectly, to prevent *wg* from activating *dac*, or represses *dac* expression directly (see Discussion).

In contrast to the activation seen in the female, *wg*-expressing clones in males are associated with the repression of *dac* when a *wg*-expressing clone approaches or overlaps with the endogenous male *dac*-expressing domain (data not shown). Moreover, male discs with near-ubiquitous *arm** expression show a severe reduction or complete elimination of *dac* expression (Fig. 5E,F). This effect is cell-autonomous: one such *arm**-expressing male disc contained a wild-type patch of cells in the *dac*-expressing region; this patch of cells expresses *dac* normally (Fig. 5G, H). Thus, *wg* exhibits a sex-specific regulatory relationship with *dac* in the genital disc whereby it activates *dac* expression in females but represses *dac* expression in males.

dpp activates *dac* in males, but represses *dac* in females

To explore the regulatory relationship between *dpp* and *dac*, we examined the effect that loss and gain of *dpp* function has on *dac* expression in male and female genital discs. Loss of *dpp* function was assayed in a dpp^{discV} heteroallelic combination, dpp^{d12}/dpp^{d14}. This class of alleles retains embryonic *dpp* function but has been shown to lack enhancers that are required for *dpp* expression in imaginal discs (Spencer et al., 1982; St Johnston et al., 1990; Blackman et al., 1991). As is the case for *wg* mutants, genital discs from dpp^{d12}/dpp^{d14} larvae are smaller than wild type. Still, female dpp^{d12}/dpp^{d14} genital discs

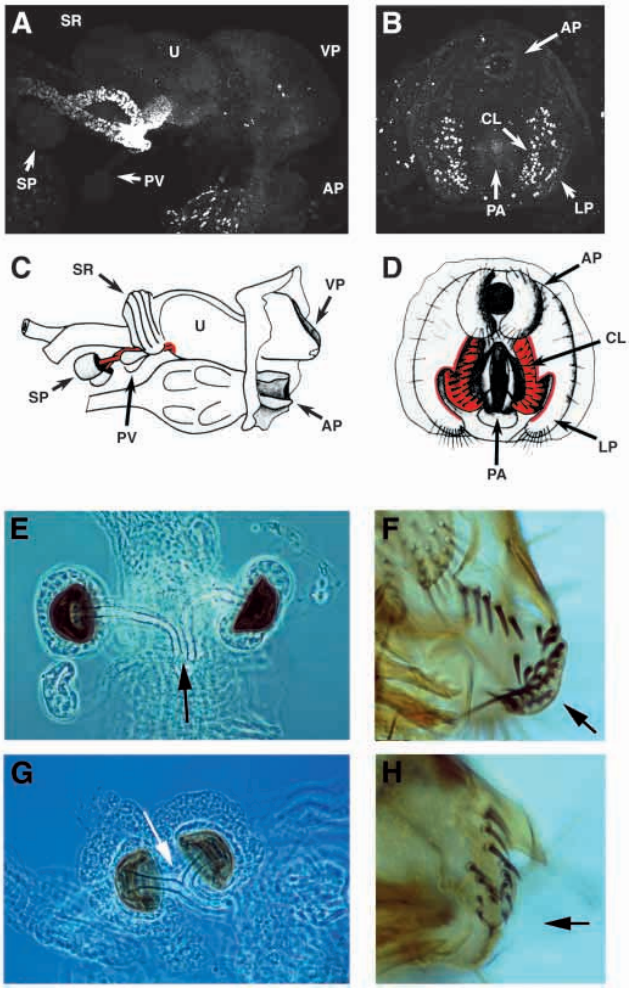

Fig. 4. *dac* is required for the proper differentiation of the genitalia. (A-D) Expression of *dac* at 48 hAPF. (A,C) Confocal image of female genitalia (A) and schematic (C) showing *dac* expression (red in schematic) in the ducts that connect the spermethecae (SP) and parovaria (PV) to the uterus (U) and in the adjacent uterine wall. (B,D) Confocal image of male genitalia (B) and schematic (D) showing *dac* expression in the developing claspers (CL) and a sliver of expression at the edge of the lateral plate (LP). SR, seminal receptacle; VP, vaginal plate; AP, anal plate; PA, penis apparatus. (E-H) The *dac* mutant phenotype. (E,G) Wild-type (E) and dac^3 (G) female internal genitalia. The ducts that connect the spermethecae to the uterus (arrow, E) have fused in the mutant (arrow, G). This phenotype was partially penetrant in dac^3 homozygotes (8/12 spermethecal ducts are fused). This is significant compared with dac^3/CyO heterozygous siblings (0/18 fused) using Fisher's exact test (P<0.0001). (F,H) Wild-type (F) and dac^3 (H) male external genitalia. The clasper (arrow, F) is severely reduced in the mutant (arrow, H). Average number of clasper teeth bristles per clasper side: dac^3 homozygotes (n=12): 15.5±1.38s.d.; dac^3/CyO (n=10): 25.1±1.72 s.d. This difference is significant (P<0.0001) using an unpaired two-tailed t-test. Schematics are adapted from Epper (Epper, 1983a) (female) and from P. C. Ehrensperger (PhD thesis, University of Zurich, 1983) (male).

express *dac* in a medial domain at levels comparable with wild type (Fig. 3J; compare with 3F). Male dpp^{d12}/dpp^{d14} genital discs, however, show very little, if any, detectable *dac* expression (Fig. 3I; compare to 3E). Thus, *dpp* is required for *dac* expression in male, but not female, genital discs. We do not reproducibly observe a lateral expansion of *dac* expression in female discs, as might be expected if lateral *dpp* expression functions to restrict *dac* to the medial domain in females. However, the presumptive *dpp* domains may simply be missing from these severely reduced discs.

In order to determine if *dpp* is sufficient, as well as necessary, to activate *dac* expression in the male genital disc, we used the flip-out GAL4 system to produce GFP-marked clones that express *dpp* ectopically. As was observed for *dpp* loss of function, ectopic *dpp* expression has a sex-specific effect on *dac* expression. In males, ectopic *dpp* can cause ectopic expression of *dac* (Fig. 6A) in and around the *dpp*-expressing clone. There does, however, seem to be a limited region of the male genital disc that is competent to activate *dac* in response to *dpp*: only clones that are in the thin dorsal epithelium anterior to the anal primordium show ectopic *dac* expression. When large numbers of small *dpp*-expressing clones are produced in the early third instar, *dac* expression can be observed to spread into a large band of cells that crosses the entire dorsal epithelium (Fig. 6B). This ectopic expression does not seem to be associated with any clone in particular, but rather seems to reflect the combined influence of the *dpp*-expressing clones that perforate the disc. We infer that this band of cells defines the region of the disc that is competent to express *dac*. A clearer result was obtained when a constitutively active form of the *dpp* receptor, *tkv** (Nellen et al., 1996) was expressed in GFP-marked clones made in the early third instar. *tkv** should activate the *dpp* signal transduction pathway, but only in the cells in which it is expressed. These small *tkv** clones can be observed to activate *dac* cell-autonomously when they occur in the *dac*-competent domain (Fig. 6C,D). The two larger clones in this figure do not activate *dac* uniformly; the portion of the clones that does not is presumably outside of the *dac*-competent domain. It is not clear why *dpp* signaling does not activate *dac* throughout the disc; there must be other factors that restrict the expression of *dac*.

In females, ectopic expression of *dpp* in the genital disc causes repression of *dac*. When large numbers of small, *dpp*-expressing clones are made in the early third instar, *dac* expression is severely reduced throughout the disc (Fig. 6E). We note that this is the same as the *wg* loss-of-function phenotype. The extent and number of *dpp*-expressing clones in these discs might be expected to block the expression of *wg*; indeed, expression detected from a *wg-lacZ* reporter carried on the same chromosome as the UAS-*dpp* transgene is severely reduced in this experiment (data not shown). This makes it difficult to determine if *dpp* is repressing *dac* by antagonizing *wg*-mediated *dac* activation in the *dac*-expressing cells, or simply by reducing the expression of *wg*. We favor the former interpretation, based on the behavior of small *tkv**-expressing clones that were made in the early third instar. These clones repress *dac* cell autonomously (Fig. 6F,G). Because the surrounding cells express *dac*, we infer that this field of cells continues to receive sufficient *wg* signal to activate *dac*. Therefore, *dpp* signal transduction can block *dac* activation even in the presence of the *wg* signal.

In addition to repressing *dac* in the female genital disc, *dpp* also appears to activate it. Careful examination of wild-type female discs shows two small regions of low-level *dac* expression in the presumptive *dpp* expression domain (data not

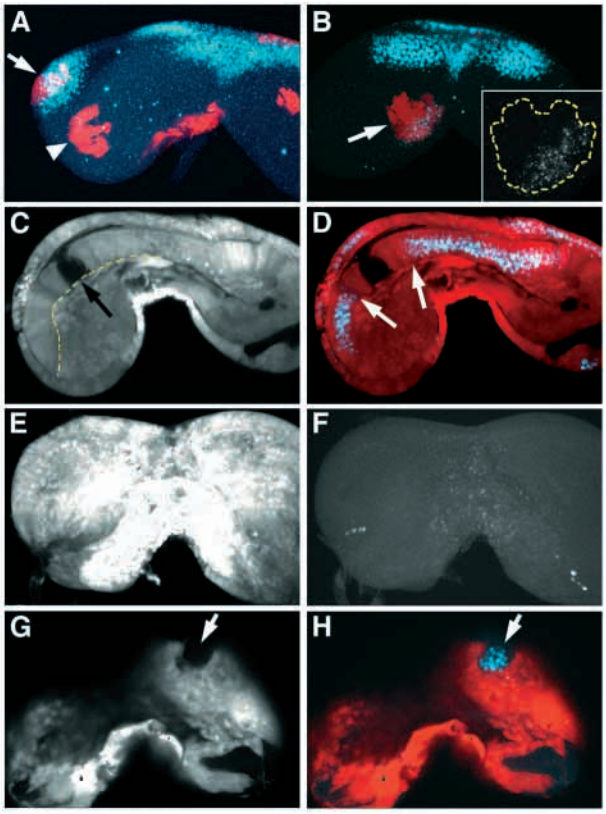

Fig. 5. Sex-specific regulation of *dac* by *wg* and *arm**. Confocal images of female (A-D) and male (E-H) genital discs in which *wg*, or the constitutively active signaling molecule *arm**, has been ectopically expressed. In all merged panels, clones, marked with GFP, are shown in red, while Dac staining is shown in light blue. (A) *wg*-expressing clones in the dorsal (RMP) region of the female disc cause ectopic expression of *dac* in and around the clone (arrow), while ventral *wg* expressing clones frequently do not (e.g. arrowhead). (B) A ventral *wg*-expressing clone in a female disc showing low level ectopic *dac* expression only on the side of the clone distal to the *dpp* expression domain. The red channel has been dimmed to allow the *dac* signal to be seen; inset, raw data from *dac* channel only, with clone outlined in yellow. The *dpp*-expressing domain is located up and to the left of the clone (compare with Fig. 3D). (C,D) A female genital disc with near-ubiquitous *arm** expression. (C) Single channel image shows the extent of *arm** expression; a small patch in the ventral epithelium does not express *arm** (arrow). Broken yellow line partially outlines the mass of adepithelial cells. (D) Disc from C, showing the resultant *dac* expression. *dac* is activated ectopically in the ventral epithelium. The patch of *arm** non-expressing cells has created a region (between the arrows) where *dac* cannot be activated by *arm**, presumably because this patch expresses *dpp*. Adepithelial cells do not express *dac*. (E-H) Near-ubiquitous expression of *arm** represses *dac* in male genital discs. (E,F) Single channel images of a male genital disc showing *arm** (E) and *dac* expression (F). *dac* expression is almost completely absent from this disc. (G,H) A male genital disc with a 'patch' of non-*arm**-expressing cells (arrow, G). Cells within this patch express *dac* (arrow, H). All clones were induced in second instar except for (B), which was induced in the first instar.

shown). Furthermore, on rare occasion we observe that ectopic *dpp* expression can activate *dac*, albeit at very low levels compared with the medial, *wg*-dependent domains (data not shown). This activation is barely detectable, but is reproducible. We also noticed that in *wg* mutant female discs, some of the remnant *dac* expression is in the presumptive *dpp*-expressing region (Fig. 3H, arrowheads). We suspect that this expression reflects a vestigial ability of *dpp* to activate *dac* that has been over-ridden in the female genital disc (see Discussion).

The sex determination pathway acts cell autonomously to modulate *dac* regulation by *wg* and *dpp*

There are two possible mechanisms that could account for the sex-specific regulation of *dac* by *wg* and *dpp* in the genital disc. In one scenario, the sex determination pathway acts directly in each cell to modulate *dac* regulation. However, the male and female primordia derive from different abdominal segments; the identity of these segments is controlled by transcription factors from the bithorax complex (Casares et al., 1997; reviewed by Jurgens and Hartenstein, 1993). Thus, an alternative hypothesis is that the genes that control segmental identity modulate *dac* regulation by *wg* and *dpp*. The results look 'sex specific' because only one segmental primordium is allowed to develop in each sex. To distinguish between these possibilities, we changed the genetic sex of cells in GFP-marked clones by using the flip-out GAL4 system to manipulate components of the sex-determination pathway. We shall distinguish between the chromosomal sex of an organism or genital disc (XX versus XY) and the genetic sex of cells as controlled by the sex determination pathway.

We generated genetically female clones in the context of a chromosomally male genital disc by ectopically expressing a female-specific *tra* cDNA (Ferveur et al., 1995); we shall refer to such clones as tra^+ clones. Ubiquitous expression of a female *tra* cDNA transforms XY males into females (McKeown et al., 1988). As the somatic sex determination pathway acts cell-autonomously (Baker and Ridge, 1980), we reasoned that if the sex of a cell determines its regulation of *dac*, then we should see a cell-autonomous variation of *dac* expression in adjacent male and female cells at the clone border. Bearing out this prediction, tra^+ clones in the male genital disc have a dual behavior, depending on their location within the disc. Where these clones extend laterally into the endogenous male *dac* domain, they appear to repress *dac* expression cell autonomously (Fig. 7B): male cells adjacent to the clone express *dac* normally, but the female cells within the clone are unable to do so. However, when such a clone extends medially, towards the source of the *wg* signal, the female cells begin to express *dac* cell-autonomously within the clone, while the adjacent male cells cannot (Fig. 7A). We infer that these genetically female tra^+ cells are unable to activate *dac* laterally in response to *dpp* but, like their counterparts in a female genital disc, activate *dac* medially in response to *wg* signaling. We interpret these results to mean that in the male primordium (A9), it is indeed genetic sex and not segmental identity that determines how *dac* is regulated. The behavior of tra^+ clones in the male genital disc offers no insight as to how *dac* is regulated in the female (A8) primordium. *dac* is not expressed in the RFP in the male disc, and we did not observe any tra^+ clones that activate *dac* there. As expected, tra^+ clones have no effect on *dac* expression in the female genital disc (data not shown).

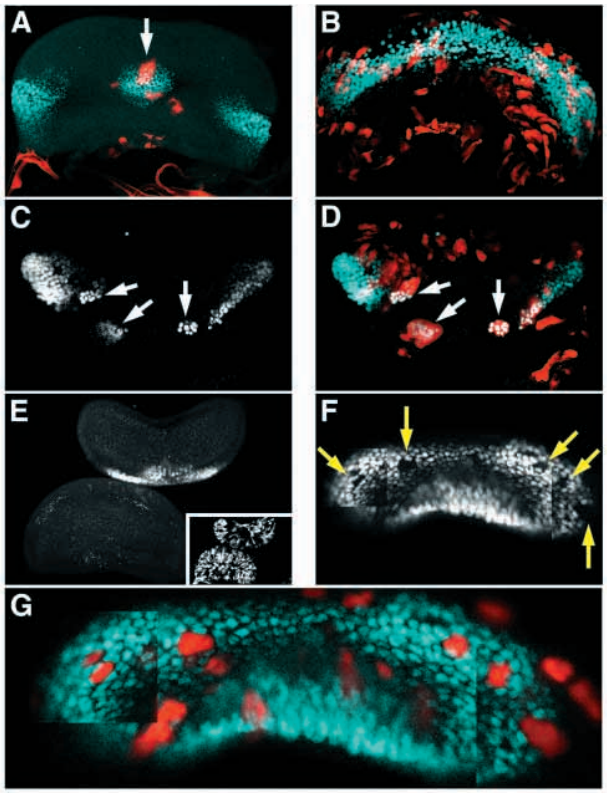

Fig. 6. Sex-specific regulation of *dac* by *dpp* and *tkv**. Confocal images of male (A-D) and female (E-G) genital discs in which *dpp*, or the constitutively active *dpp* receptor *tkv**, has been ectopically expressed. In all merged panels, clones, marked with GFP, are shown in red, while *dac* staining is shown in light blue. (A) A second instar *dpp*-expressing clone in the male genital disc can cause ectopic activation of *dac* (arrow) in the dorsal epithelium. Note that the expression of *dac* is non-uniform around the clone. Clones induced in first and third instar had similar effects. (B) A male genital disc with multiple third instar *dpp*-expressing clones shows activation of *dac* in a band across the dorsal epithelium. This band may define the '*dac* competent' region. (C,D) Third instar *tkv**-expressing clones can activate *dac* in the dorsal epithelium when they intersect the '*dac*-competent' region. *dac* expression (C) and merge (D) showing ectopic *dac* caused by *tkv**-expressing clones (arrows). Note that *dac* expression is non-uniform within two of the clones. (E) *dac* expression in sibling female genital discs, one of which (bottom) carries the UAS-dpp transgene. Inset: GFP channel shows that both discs are perforated by UAS-GFP-expressing clones. The UAS-dpp carrying disc shows almost complete loss of *dac* expression, while *dac* expression in the control disc (top) is normal. (F,G) A female genital disc containing small third instar *tkv**-expressing clones. (F) *dac* expression in a composite of several confocal slices shows holes of repression in the dorsal *dac* domain (arrows). (G) Merge of GFP-channel shows 1:1 correspondence of clones and *dac* repression.

To address whether *dac* is regulated by the sex determination pathway or segmental identity in the female primordium, we created genetically male clones within the female genital disc and assessed their effects on *dac* expression. We availed ourselves of a new technique, which combines the flip-out

GAL4 system with dsRNA-mediated interference (reviewed in Bosher and Labouesse, 2000), to generate clones in which the function of the *tra-2* gene is disrupted. In these clones, GAL4 activates transcription of a transgene in which an inverted repeat of the *tra-2* locus has been placed under the control of the UAS promoter. When expressed ubiquitously at high levels throughout the fly, this construct produces a strong *tra-2* loss-of-function phenotype, transforming XX females into somatic males (Fortier and Belote, 2000). We shall refer to clones that express the *tra-2* inverted repeat as *tra2IR* clones. If the sex determination pathway controls *dac* regulation in the female genital disc as well as the male disc, we expect *tra2IR* clones to switch from the female to the male mode of *dac* regulation. That is, they should repress *dac* in the medial, *wg*-dependent domain, but activate *dac* laterally where *dpp* is expressed.

The behavior of genetically male *tra2IR* clones in chromosomally female genital discs depends on whether they are made in the repressed male primordium (A9) or in the female primordium (A8). In the RMP, *tra2IR* clones cause large outgrowths that, when of sufficient size, begin to take on the morphology of the male genital disc. This mirrors a similar result obtained when *tra* function is removed by mitotic recombination (Epper and Nothiger, 1982), supporting our interpretation that these clones are switching to the male mode of differentiation. *dac* expression in these clones has a dual behavior: Where such clones extend medially, into the *wg*-dependent *dac* domain, they appear to repress *dac* (Fig. 7C). However, clones that extend laterally, into the region of the disc where *dac* is expressed in males, can activate *dac* expression (Fig. 7D). Large clones that span these two regions exhibit both types of behavior simultaneously. We interpret these results to mean that genetically male cells cannot express *dac* in response to *wg* but do express *dac* when provided with the *dpp* signal. This result is precisely the inverse of that obtained when tra^+ clones were made in the male genital disc, and corroborates our conclusion that in the male (A9) primordium, the sex determination pathway determines how *dac* is regulated by *wg* and *dpp*.

The behavior of *tra2IR* clones in the female primordium (A8) of a chromosomally female disc is less informative. These clones are never observed to activate *dac* in the female primordium, even though they might grow to be quite large and encompass much of the presumptive *dpp*-expressing domain (Fig. 7D). Only a few *tra2IR* clones are observed to extend medially into the *wg*-dependent *dac* domain, and these repress *dac* expression (data not shown). The observed repression of *dac* could be interpreted to mean that genetically male A8 cells are unable to express *dac* in response to *wg*. However, it is important to note that the fate of A8 cells in a genetically male fly is to become part of the repressed female primordium (Nothiger et al., 1977; Schupbach et al., 1978; Epper and Nothiger, 1982; Wieschaus and Nothiger, 1982). Accordingly, the male cells in these clones might be adopting a generally non-responsive state similar to that of the RFP. These results do not allow us to determine whether the sex determination pathway, or segmental identity, modulates *dac* regulation by *wg* and *dpp* in A8.

doublesex is the likely mediator of sex-specific *dac* regulation

dsx is the most downstream regulatory gene in the branch of the somatic sex determination hierarchy that controls genital disc development, and encodes male- and female-specific transcription factors (Baker and Ridge, 1980; Burtis and Baker, 1989; Erdman and Burtis, 1993). Thus, *dsx* is the most likely candidate to mediate sex-specific *dac* regulation by *wg* and *dpp*. On the basis of our results with tra^+ and *tra2IR* clones, we can make predictions as to how Dsx^m and Dsx^f might regulate *dac*. For example, tra^+ clones in a chromosomally male genital disc stop expressing the Dsx^m isoform and begin expressing the Dsx^f isoform. Concomitantly, such clones repress (or lose) *dac* expression laterally and activate *dac* expression medially. Therefore, *dac* expression in the lateral domain might require Dsx^m, be repressed by Dsx^f, or both. Conversely, *dac* activation in the medial domain might require Dsx^f, be repressed by Dsx^m, or both.

In order to distinguish between these possibilities, we

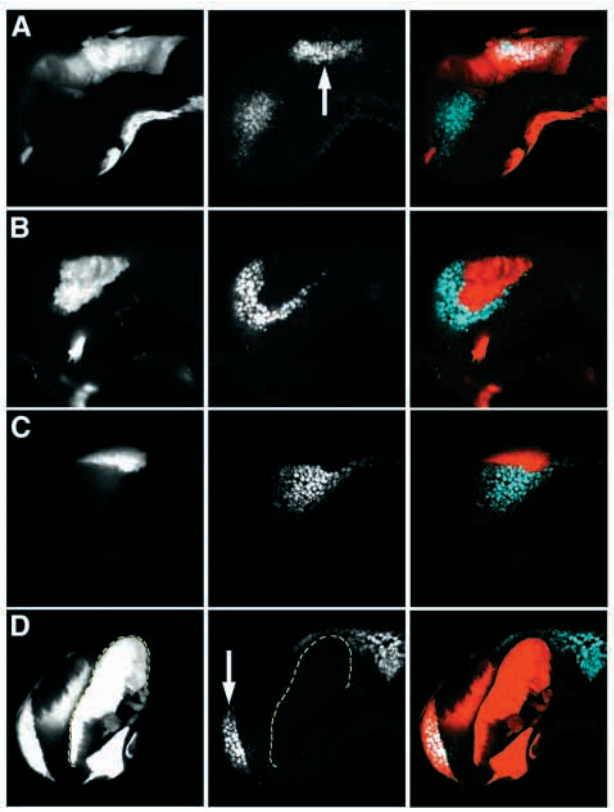

Fig. 7. The sex determination pathway determines *dac* regulation. Confocal images of genital discs showing GFP-marked clones (left), *dac* expression (middle), and merged images (right). (A,B) tra^+-expressing clones in male genital discs. (A) A clone extending medially activates *dac* expression ectopically within the clone (arrow). (B) A clone extending laterally into the endogenous male *dac* domain causes repression of *dac* cell-autonomously. (C,D) A female genital disc containing several large *tra2IR*-expressing clones. One clone in the RMP has caused a large outgrowth; another large clone takes up a significant fraction of the ventral female primordium. (C) The RMP clone extends medially into the endogenous *dac* domain and represses *dac* cell-autonomously. (D) The same RMP clone activates *dac* where it extends laterally (arrow). The large clone in the female primordium (broken yellow line) does not activate *dac*, even though it occupies much of the presumptive *dpp*-expressing region. All clones were induced in the first larval instar.

examined the effect of *dsx* loss of function on *dac* expression. Genital discs from XX females of the genotype dsx^{D+R3}/dsx^{M+R15} were dissected and stained with anti-Dac monoclonal antibody. This allelic combination is a molecular null for *dsx* (Taylor, 1992), and both the male and female primordia of an XX dsx^{D+R3}/dsx^{M+R15} genital disc develop. In the male primordium of these intersexual discs, we observe ectopic activation of *dac* in a medial domain (Fig. 8B), while the lateral *dac* expression domains appear normal. Thus, we conclude that in the male primordium Dsx^m is not required to activate *dac* expression in the lateral domain, and therefore the loss of *dac* expression laterally in tra^+ clones must be due to repression by Dsx^f. Furthermore, the ectopic medial expression of *dac* suggests that Dsx^m is required to repress activation of *dac* medially in the male primordium and that this activation does not require Dsx^f.

With respect to *dac* regulation in the female primordium, our results are ambiguous. Sometimes *dac* expression is completely absent from the female primordium of dsx^{D+R3}/dsx^{M+R15} mutant genital discs; in other instances there is a reduced medial domain that expresses *dac* at wild-type levels (Fig. 8A, arrow). We never observed ectopic activation of *dac* laterally in the female primordium, which would be expected if Dsx^f were required to repress *dac* expression there. This is consistent with the result that *tra2IR* clones, which switch expression from Dsx^f to Dsx^m, do not cause ectopic lateral activation of *dac* in the female primordium. The results would seem to imply that Dsx^f is partially required for the medial (*wg*-activated) expression of *dac* in the female primordium. However, the female primordium develops to variable extent in *dsx* mutants, and thus the reduced *dac* expression might reflect a general retardation of the development of the primordium. We favor the interpretation that Dsx^f is not required for medial activation of *dac* in the female primordium.

DISCUSSION

Sex-specific gene regulation in the genital disc

One important unanswered question concerning the genital disc has been whether the sex determination pathway, and *dsx* in particular, plays an instructive or permissive role in its development and differentiation. In this work we show that in some respects, this role is demonstrably instructive. The sex determination pathway mediates the sex-specific deployment of a gene, *dac*, to specific regions of the genital disc, where it is required for the differentiation of adult structures. Intriguingly, the sex determination pathway achieves this feat by modulating the regulation of *dac* by *wg* and *dpp*, two genes whose function is to establish positional identity within the disc. Modulation of existing regulatory interactions may prove to be a general strategy for producing sexual dimorphism.

dac regulation by *wg* and *dpp*

We have shown that *dac* is sex-specifically regulated by *wg* and *dpp* in the genital disc. In the female genital disc *wg* activates, and *dpp* represses, *dac*. *dac* expression in the female genital disc correlates with *wg* expression in both the ventral female primordium and the dorsal RMP. Further, *wg* mutant female genital discs lose *dac* expression, while *dpp* mutant female discs do not. Finally, female genital discs respond to ectopic *wg* or *arm** by activating *dac*, while ectopic expression of *dpp* represses *dac*. Thus, *wg* signaling is both necessary and sufficient for *dac* expression in the female disc.

It could be argued that repression of *dac* by *dpp* in the female disc is simply an indirect result of the ability of *dpp* to downregulate *wg* expression. Based on the following evidence, we favor the interpretation that the repression of *dac* by *dpp* in the female disc does not act at the level of *wg* expression. If *dpp* represses *dac* solely by preventing *wg* expression, then this repression should be over-ridden by ectopic expression of *wg* or *arm**, as these are expressed from a promoter that *dpp* cannot regulate. We observe instead that *wg* and *arm** can activate *dac* only in certain regions of the disc. This non-uniform response of *dac* to *wg* expression or *arm** can be explained, at least in part, by the ability of *dpp* to antagonize *wg*-mediated *dac* activation. Near-ubiquitous expression of *arm**, a condition that would be expected to repress *dpp*, allows *dac* to be activated ectopically by *arm** in the ventral female disc. However, a patch of wild-type cells in the presumptive *dpp*-expressing domain caused a 'halo' of *dac* repression that extends into the *arm**-expressing cells. We interpret this halo of repression to be the result of Dpp that is diffusing from this wild-type patch. Finally, small *tkv**-expressing clones are observed to repress *dac* expression cell-autonomously in the female genital disc. *dac* expression remains intact in the surrounding cells, arguing that these clones continue to receive the *wg* signal. Our results cannot distinguish whether *dpp* represses *dac* by acting directly on *dac* regulatory regions, or by interfering with *wg* signal transduction generally. We note that Brook and Cohen reported that the ability of *wg* to activate the *H15* gene in the leg was antagonized by endogenous *dpp* expression (Brook and Cohen, 1996). They observed a non-uniform response of *H15* around a patch of ectopic *wg* expression: *H15* was expressed only on the side of the *wg*-expressing patch that was distal to the endogenous *dpp* domain. This is strikingly similar to the results we observed in ventral *wg*-expressing clones in the female genital disc.

In the male genital disc, *dpp* activates *dac* while *wg* represses it. First, *dac* expression partially overlaps the two lateral *dpp*-expressing domains. Further, *dpp* mutant male genital discs lose *dac* expression, while in *wg* mutant male discs, *dac* expression actually expands across the disc. Finally, ectopic expression of *dpp* and the activated *dpp* receptor *tkv** can cause

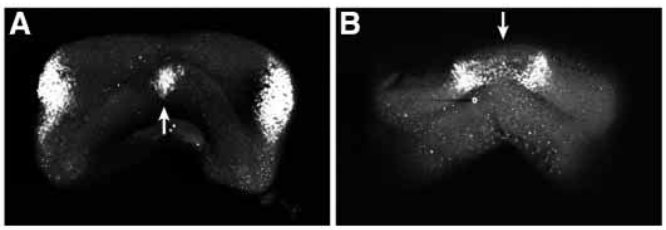

Fig. 8. *dsx* is required for proper *dac* regulation in the male primordium. Confocal images showing *dac* expression in an XX dsx^{D+R3}/dsx^{M+R15} mutant genital disc. (A) Confocal section through the male *dac* expression domains, showing that they are normal. Note the small patch of *dac* expression in the female primordium (arrow). (B) A projection of several confocal sections from the ventral region of the male primordium, showing ectopic medial expression of *dac* (arrow).

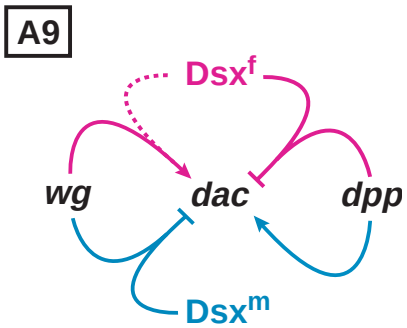

Fig. 9. A model for combinatorial *dac* regulation in the male primordium. The evidence suggests that both *wg* and *dpp* are capable of activating *dac* in the male primordium in the absence of *dsx* function (see text). Sex-specific regulation of *dac* in the female genital disc (pink) occurs when Dsx^f turns *dpp* into a repressor of *dac* and possibly potentiates activation by *wg*. In the male genital disc (blue), Dsx^m turns *wg* into a repressor of *dac* but is not required for activation of *dac* by *dpp*.

ectopic expression of *dac* in the male disc, while ectopic expression of *wg* or *arm** represses *dac*. Ectopic *dpp* and *tkv** can only activate *dac* in a small '*dac*-competent' region of the dorsal epithelium in the male, leading us to suspect that there are additional factors that regulate *dac*.

It is not clear whether *wg* represses *dac* in the male disc directly or by antagonizing *dpp*-mediated *dac* activation. From a formal genetic standpoint, the expansion of *dac* into the medial domain of a male *wg* mutant genital disc would argue that *wg* represses *dac*. However, it has been shown that loss of *wg* function can allow *dpp* expression to invade the presumptive *wg*-expressing domain (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). Thus, the observed expansion of *dac* expression could reflect the expansion of the *dpp*-expressing domain. Nevertheless, we favor the interpretation that *wg* regulates *dac* in the male genital disc in a manner independent of its antagonism of *dpp* function. A precedent for such a regulatory relationship exists: the male primordium, in its incarnation as the RMP of a female disc, expresses *dac* in response to *wg*. We note also that the ectopic medial expression of *dac* in *dsx* mutant discs correlates with *wg* expression, implying that in the absence of Dsx^m, *wg* activates *dac* in the male genital primordium.

It is interesting to consider *dac* regulation in light of the evolution of the genital disc. The single, bilaterally symmetric genital disc is thought to have evolved from paired genital discs that fused across the ventral midline (Gleichauf, 1936; Dubendorfer, 1971; Emmert, 1972). Pattern formation may have been accomplished in these paired discs in much the same way that it is in the leg imaginal disc. This hypothesis is supported by comparative studies of pattern formation genes and their regulatory interactions in the leg and genital discs, which show that the essential regulatory relationships are preserved (Chen and Baker, 1997; Casares et al., 1997; Sanchez et al., 1997; Gorfinkiel et al., 1999). Thus, the regulation of *dac* in the leg disc provides a context for the interpretation of our results. In the leg, *wg* and *dpp* cooperate to produce the mid-proximal ring of *dac* expression. Intermediate levels of Wg and Dpp synergize to activate *dac*, while high levels of Wg and Dpp together repress *dac* expression (Lecuit and Cohen, 1997). It is tempting to speculate that this regulatory relationship has been modified to produce that observed in the genital disc. This is consistent with the observation that in the male primordium of XX *dsx* discs, *dac* is expressed in both lateral, *dpp*-dependent domains and in a medial, presumably *wg*-dependent domain. In further support of this hypothesis, we note that in the female genital disc, there is a barely detectable level of *dac* expression in the presumptive *dpp*-expressing domain, and a low level of Dac remains in the lateral regions of female discs from *wg* mutant larvae. Moreover, ectopic *dpp* in the female disc was occasionally observed to activate *dac* at very low levels, in addition to repressing it. This may reflect a vestigial ability of *dpp* to activate *dac* in the female genital primordium that has been suppressed during the evolution of the genital disc.

dac regulation by the sex determination pathway

A number of obstacles make it difficult to demonstrate that the sex determination pathway is responsible for the sex-specific regulation of a gene in the genital disc. These obstacles stem from the fact that the male and female primordia, which are the primary constituents of their respective discs, differ in their segmental origin (Nothiger et al., 1977; Schupbach et al., 1978; Epper and Nothiger, 1982). This raises the possibility that 'sex-specific' gene regulation is really just segment-specific gene regulation, made to look sex specific by the fact that only one primordium develops in each sex. We attempted to address this concern by creating clones of the opposite genetic sex in chromosomally male and female genital discs. Thus, for example, we were able to examine *dac* regulation in the male (A9) primordium, in both male and female cells. By varying the genetic sex of cells in a context where segmental identity is uniform, we hoped to disentangle the contributions of sex and segmental identity to *dac* regulation.

In the male primordium of both male and female discs, the regulation of *dac* varies according to the genetic sex of the cell. Genetically female clones in the male (A9 derived) primordium of the male genital disc are unable to express *dac* in the lateral male (*dpp*-dependent) domain, but are able to express *dac* when they extended medially, towards the source of Wg. Conversely, in the female genital disc, genetically male clones in the repressed male primordium (A9) lose their ability to express *dac* in the medial, *wg*-dependent domain, and begin to express *dac* laterally, presumably in response to Dpp. Finally, *dac* expression is abnormal in intersexual genital discs from *dsx* mutant larvae: The male primordium of *dsx* genital discs expresses *dac* in both the endogenous, lateral male domains, and in a slightly weaker medial domain that corresponds roughly to the region where tra^+ clones are able to activate *dac*. Thus, we conclude that, in the male primordium, the sex determination pathway determines how a cell will regulate *dac*.

In the female primordium our results fail to show a role for the sex determination pathway in *dac* regulation. If such a role exists, we would expect that genetically male clones in the female primordia of a female genital disc would activate *dac* laterally, like their counterparts in the male primordia. They do not, even when they take up much of the presumptive *dpp*-expressing domain. We would also expect such clones to repress *dac* medially. Only a few clones were observed to

extend into the medial *wg*-expressing domain, and as expected these appear to repress *dac*. Interpretation of these results is complicated by the fact that changing the genetic sex of a cell in the genital disc can cause it to enter the 'repressed' state (Epper and Nothiger, 1982; Wieschaus and Nothiger, 1982). Thus, for example, if a genetically male clone represses *dac* when it intersects the medial *dac* domain in the female primordia, we can conclude either that the sex determination pathway regulates *dac* expression or that the cells, which are now male, have adopted a repressed state and are generally unresponsive. A similar caveat prevents us from interpreting the failure of *tra2IR* clones to activate *dac* ectopically in the female primordium. We are not concerned that tra^+ clones in the male primordium of male genital discs enter such a generally non-responsive state, because these clones both repress *and* activate *dac* expression. The expression pattern of *dac* in the female primordium of a *dsx* mutant genital disc is also difficult to interpret. *dac* is not activated ectopically in the lateral domains of the *dsx* female primordium, which is consistent with the failure of *tra2IR* clones to cause such activation. However, even the medial, *wg*-dependent *dac* domain is frequently absent or severely reduced in the *dsx* female primordium, and thus we are reluctant to draw any conclusions from the absence of ectopic *dac* laterally.

We propose a model (Fig. 9) for *dac* regulation in the male primordium, in which the different isoforms of Dsx protein modulate *dac* regulation by *wg* and *dpp*. In the absence of *dsx*, both *wg* and *dpp* can activate *dac*, producing the two domains of *dac* expression observed in the male primordium of a *dsx* disc. In the female, Dsx^f modulates *dpp* activity so that *dpp* becomes a repressor of *dac*; Dsx^f may also potentiate the activation of *dac* by *wg*. In the male, Dsx^m modulates *wg* activity so that it becomes a repressor of *dac*, leaving *dpp* alone to activate *dac*. In support of this model, we note that the Dsx proteins act in a similar manner to positively or negatively modulate the effect of tissue-specific regulators on the *yp* genes (An and Wensink, 1995a; An and Wensink, 1995b).

On the nature of 'repression' in the undeveloped genital primordium

The behavior of tra^+ and *tra2IR* clones provides insight into the mechanism of repression in the undeveloped genital primordium. We had anticipated that such clones would be difficult to recover when they occurred in the male and female primordium, respectively, because they should adopt the repressed state (Epper and Nothiger, 1982; Wieschaus and Nothiger, 1982). Instead, we recovered large tra^+ (female) clones in the male primordium of a male disc, and large *tra2IR* (male) clones in the female primordium of a female disc. Some of these clones constitute a substantial fraction of the primordium in question. Though we did not score tra^+ or *tra2IR* clones in adults, previous studies strongly suggest that such clones would fail to differentiate adult genital structures. Wieschaus and Nothiger showed that tra^- (male) clones caused large deletions in the female genitalia (Wieschaus and Nothiger, 1982), indicating that genetically male cells like those in a *tra2IR* clone divide but cannot differentiate female genital structures. Further, when Schupbach et al. analyzed the genitalia of gynandromorphs, they found that male structures were deleted when the mosaic border passed through the male genitalia (Schupbach et al., 1978), suggesting that female tissue cannot differentiate male structures. To reconcile these data, we propose that repression of the inappropriate genital primordium involves two separable processes: repression of growth and the prevention of differentiation. Thus, clones of cells of the inappropriate genetic sex cannot differentiate, but they can grow and contribute to a morphologically normal genital primordium.

This poses yet another question. Cells in a tra^+ clone in the male primordium of a male genital disc are analogous to the cells in the repressed male primordium of a wild-type female genital disc: both are genetically female, and both have A9 segmental identity. Why do tra^+ clones in the male primordium grow, while the repressed male primordium in a female disc does not? One possibility is that the decision of the male primordium to grow in a male disc is made before tra^+ clones were induced and cannot be over-ridden by a later switch of genetic sex. However, temperature-shift experiments with $tra\text{-}2^{ts}$ alleles suggest that the decision of a genital primordium to develop can be reversed later in development (Belote and Baker, 1982; Sanchez and Granadino, 1992). Furthermore, occasional, large tra^+ clones can cause severe reductions in male genital discs (data not shown). This observation leads us to suggest a model in which growth in the genital disc is regulated from within organizing zones, such as the domains of *wg* and *dpp* expression. According to this model, the sex of the cells in the organizing regions would determine how the disc grows, while cells in other regions would respond accordingly, regardless of their sex. The tra^+ clones that cause reduction could result when such a clone intersects with one of the postulated organizing centers within the disc. The implication is that the sex determination pathway acts in yet undiscovered ways to modulate the function of the genes that establish pattern in the genital disc. We have found one such interaction in the regulation of *dac*; further study is needed to determine if others exist, and what role they play in producing the sexual dimorphism of the genital disc and its derivatives.

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