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 (tra2; tra2 – FlyBase) locus, Tra directs the female-specific splicing of the dsx pre-mRNA, producing an mRNA that encodes the DsxL 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\textsuperscript{m} protein. \textit{dxx} 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 Burris, 1993). The only genes that are known to be directly regulated by the Dsx proteins are the yolk protein genes \textit{yp1} and \textit{yp2} (\textit{Yp1} and \textit{Yp2} – FlyBase). The \textit{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 \textit{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 Dsx proteins, both male and female Dsx proteins are required for proper \textit{yp} gene regulation: \textit{Dsx}\textsuperscript{f} activates transcription of the \textit{yp} genes in females and \textit{Dsx}\textsuperscript{m} represses their transcription in males (Coschigano and Wensink, 1993). Evidence suggests that regulation by \textit{dxx} 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 \textit{dxx} plays a permissive or an instructive role in the differentiation of the genitalia. In the absence of \textit{dxx} 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 \textit{dxx} 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 \textit{dxx} 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 \textit{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 \textit{dxx} in genital disc development might be gained by the discovery of genes whose expression in the genital primordia is regulated by \textit{dxx}.

One approach to identifying potential \textit{dxx} 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 \textit{dxx} 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 \	extit{engrailed (en)} gene. As in the leg disc, \textit{en} acts through the secreted protein encoded by \	extit{hedgehog (hh)} to de-repress \textit{wingless (wg)} and \textit{decapentaplegic (dpp)} in complementary and mutually exclusive domains along the anterior/posterior (A/P) border. \textit{wg} and \textit{dpp}, in turn, encode secreted morphogens that specify positional information (Belote, 1989; Klingensmith and Nusse, 1994; Lecuit et al., 1996; Nellen et al., 1996). While the paired leg discs each possess a dorsal stripe of \textit{dpp} expression and a ventral stripe of \textit{wg} expression, the bilaterally symmetric, unpaired genital disc has a single, medial \textit{wg} expression domain that is flanked by two lateral \textit{dpp} expression domains. The mutually exclusive domains of \textit{wg} and \textit{dpp} expression are maintained by a system of mutual antagonism: in the leg disc, for example, ventral \textit{wg} expression represses \textit{dpp} expression to confine it dorsally; \textit{dpp}, in turn, represses \textit{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 \textit{dachshund (dac)} gene, a known target of \textit{wg} and \textit{dpp} regulation in the leg disc, is expressed sex specifically in the genital disc. In the leg disc, \textit{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 \textit{Wg} and \textit{Dpp} proteins co-operate to activate \textit{dac} in the mid-proximal leg, while high levels of \textit{Wg} and \textit{Dpp} in turn repress \textit{dac}, excluding \textit{dac} expression from the distal leg (Lecuit and Cohen, 1997). Other genes such as \textit{homothorax (ho)} refine this regulation in order to bring about the final pattern of \textit{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, \textit{dac} is activated by \textit{dpp} and repressed by \textit{wg}, while in female genital discs, the converse relationship exists. This results in the sex-specific deployment of \textit{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 \textit{wg}, \textit{dpp} and \textit{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-\textit{wg} (H. Krause, unpublished); UAS-\textit{tkv}\textsuperscript{*} (Nellen et
Clones that ectopically express dpp were generated in larvae of the following genotypes: y w hsflp1/yw; UAS-dpp/Actin>y-GAL4, UAS-GFP; UAS-tra2/IR (see Fig. 6A); Actin>CD2>GAL4; UAS-dpp, wg-lacZ/UAS-GFP; MKRS, hsFLP/+ (see Fig. 6B); Actin>CD2>GAL4/y w hsflp1; 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-tra2-IR; UAS-GFP/+; MKRS, hsFLP /UAS-tra2-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-tra2-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 Notherg, 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 (hAE), 37°C × 30 minutes (see Fig. 5A); 24-48 hAE, 37°C × 30
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 male primordium (RFP) is clearly set aside at the ventral posterior end of the epithelium of the female disc, the repressed female primordium retains its position at the posterior of the dorsal epithelium and a thin dorsal epithelium. The anal genital disc, the male primordium develops to produce a highly columnar ventral epithelium consisting 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 male primordium (RFP) is clearly set aside at the ventral posterior end of the epithelium 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; Gorflinkiel 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 swath 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 spermathecae 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

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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 DsxM. DsxL 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 DsxM. DsxM 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 spermathecae 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 spermathecae, but the two ducts are fused into one branched duct that is shared by both spermathecae (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 (Gorfinkel 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 effect 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 $wg$ in the male genital disc (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) Larval genital discs from $dpp^{112}/dpp^{114}$ 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.

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) Larval genital discs from $dpp^{112}/dpp^{114}$ 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.

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
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*-expressing 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<sup>discV</sup> heteroallelic combination, dpp<sup>d12/dpp<sup>d14</sup></sup>. 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<sup>d12/dpp<sup>d14</sup></sup> larvae are smaller than wild type. Still, female dpp<sup>d12/dpp<sup>d14</sup></sup> genital discs
express *dac* in a medial domain at levels comparable with wild type (Fig. 3J; compare with 3F). Male *dpp* 

*het1/*dpp* 

14 *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 shown). These clones that were made in the early third instar. These clones interpret, 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.

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 *dpp* 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 adependithelial 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*. Adependithelial 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 (Casareis 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).

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 Boshier and Labosse, 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. 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 dsxD+/R15/dsxM+/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 dsxD+/R15/dsxM+/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 DsxM+ is not required to activate dac expression in the lateral domain, and therefore the loss of dac expression laterally in tra2IR clones must be due to repression by DsxR. Furthermore, the ectopic medial expression of dac suggests that DsxM+ is required to repress activation of dac medially in the male primordium and that this activation does not require DsxR.

With respect to dac regulation in the female primordium, our results are ambiguous. Sometimes dac expression is completely absent from the female primordium of dsxD+/R15/dsxM+/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 DsxR were required to repress dac expression there. This is consistent with the result that tra2IR clones, which switch expression from DsxR to DsxM, do not cause ectopic lateral activation of dac in the female primordium. The results would seem to imply that DsxR 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 DsxR 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 dsxD+/R15/dsxM+/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).
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 and possibly potentiates activation by wg. In the male genital disc (blue), Dsx* turns wg into a repressor of dac but is not required for activation of dac by dpp.

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 dxx function (see text). Sex-specific regulation of dac in the female genital disc (pink) occurs when Dsx* turns dpp into a repressor of dac and possibly potentiates activation by wg. In the male genital disc (blue), Dsx* turns wg into a repressor of dac but is not required for activation of dac by dpp.

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 dxx 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 dxx mutant larvae: The male primordium of dxx 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 genetic 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, \( Dsx^\alpha \) modulates \( dpp \) activity so that \( dpp \) becomes a repressor of \( dac \); \( Dsx^\beta \) may also potentiate the activation of \( dac \) by \( wg \). In the male, \( Dsx^\alpha \) 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 \( tra-2^a \) 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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