Analysis of the Doublesex Female Protein in *Drosophila melanogaster*: Role in Sexual Differentiation and Behavior and Dependence on Intersex

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ABSTRACT

doublesex (dsx) is unusual among the known sex-determination genes of *Drosophila melanogaster* in that functional homologs are found in distantly related species. In flies, *dsx* occupies a position near the bottom of the sex determination hierarchy. It is expressed in male- and female-specific forms and these proteins function as sex-specific transcription factors. In the studies reported here, we have ectopically expressed the female Dsx protein (Dsx^F) from a constitutive promoter and examined its regulatory activities independent of other upstream factors involved in female sex determination. We show that it functions as a positive regulator of female differentiation and a negative regulator of male differentiation. As predicted by the DNA-binding properties of the Dsx protein, Dsx^F and Dsx^M compete with each other for the regulation of target genes. In addition to directing sex-specific differentiation, Dsx^F plays an important role in sexual behavior. Wild-type males ectopically expressing Dsx^F are actively courted by other males. This acquisition of feminine sex appeal is likely due to the induction of female pheromones by Dsx^F. More extreme behavioral abnormalities are observed when Dsx^F is ectopically expressed in dsx^- XY animals; these animals are not only courted by, but also copulate with, wild-type males. Finally, we provide evidence that *intersex* is required for the feminizing activities of Dsx^F and that it is not regulated by the sex-specific splicing cascade.

MECHANISMS that govern the choice of sexual identity are often separate and distinct from the actual differentiation process that ultimately gives rise to sex-specific morphology, behavior and gametogenesis. This separation of choice and differentiation has given rise to the idea that the evolution of sex determination might be a reverse-order process (Wilkins 1995). In this model, upstream regulators that determine the choice of sexual identity would undergo rapid change as new species are formed. As a consequence, the genes and/or the mechanisms that determine the choice of sexual identity would tend to be quite divergent. In contrast, downstream genes that are responsible for directing the different aspects of sexual differentiation would tend to be conserved across species and belong to a common collection of ancestral genes with roughly similar functions.

This hypothesis is consistent with what is known about the well-studied sex determination pathway in the fruit fly *Drosophila melanogaster* (Baker 1989; Cline and Meyer 1996). The choice of sexual identity in *melanogaster* depends upon the upstream regulator gene *Sex-lethal* (Cline 1984, 1988). When *Sxl* is on, it orchestrates female differentiation by controlling several different gene cascades (Nagoshi *et al.* 1988). When *Sxl* is off, male differentiation occurs by default. While the not-too-distant house fly (Musca) also has a "*Sxl*" gene, it does not appear to have any role in sex determination (Meise *et al.* 1998). Thus, the choice of sexual identity appears to be controlled by a completely different gene hierarchy. At the opposite end of the spectrum is the downstream sexual differentiation gene *doublesex* (*dsx*). In fruit flies, Dsx is expressed in two forms, female (Dsx^F) and male (Dsx^M). Each form has positive and negative regulatory functions required for sex-specific differentiation (Burtis and Baker 1989). As predicted by the reverse-order model, Dsx protein is found in other distant species. In *Caenorhabditis elegans*, a *dsx* homolog, *mab-3*, has recently been identified (Raymond *et al.* 1998). Although *mab-3* occupies a position similar to *dsx* at the bottom of a sex-determination hierarchy, *mab-3* appears to be required only in males. Like dsx^M in flies, *mab-3* is required for the development of specialized male-specific structures and to prevent the expression of female-specific genes. Remarkably, the male, but not the female, Drosophila, Dsx protein can rescue *mab-3* mutants. The *C. elegans* Mab-3 protein has a DNA-binding domain that is quite similar to that of *dsx* and has been defined as the DM domain for ***d****sx* and ***m****ab3* (Raymond *et al.* 1998). A DM-containing gene has also been isolated in humans (DMT1 for DM domain expressed

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in testes), which maps to a chromosomal region implicated in sex reversal (Raymond *et al.* 1998). These findings suggest that the general role of *dsx* in sexual differentiation may be conserved in diverse species and that understanding the functions of this gene in flies may aid in understanding sexual differentiation in other species.

The male and female forms of the Dsx protein share a common N-terminal domain, but have different C-terminal domains. The different C-terminal domains are generated by the sex-specific splicing of the last exon of the *dsx* pre-mRNA (Burtis and Baker 1989). Sex specifically expressed Transformer protein (Tra), together with the constitutively expressed Transformer-2 protein (Tra-2), promotes the female splicing of *dsx* pre-mRNAs by activating the female 3′ splice site (Nagoshi *et al.* 1988; Hedley and Maniatis 1991; Ryner and Baker 1991; Tian and Maniatis 1994). Biochemical studies indicate that the common N-terminal domain of Dsx is responsible for DNA binding and that the male- and female-specific forms recognize precisely the same DNA sequence (An *et al.* 1996; Erdman *et al.* 1996). Also, within the common N-terminal domain is a subdomain that mediates homologous protein:protein interactions (An *et al.* 1996; Erdman *et al.* 1996; Cho and Wensink 1997). The male and female C-terminal domains are believed to function as the sex-specific regulatory elements. They also contain their own sex-specific homotypic protein:protein interaction subdomains (Cho and Wensink 1997).

Most of what is known about the *in vivo* functions of Dsx has come from studies of loss-of-function mutants and constitutive dsx^{Dom} alleles (dsx^{M}). Loss-of-function mutations affect sexual differentiation in both males and females. dsx^{-} males have an intersexual phenotype: male-specific sex-comb teeth on the foreleg are not properly formed, the abdomen is only lightly pigmented, the genitalia are malformed, and low levels of yolk protein (expressed only in females) can be detected (Baker and Ridge 1980). An intersexual phenotype is also observed in dsx^{-} females. Instead of the normal transverse row of bristles on the foreleg, incompletely formed sex-comb teeth are observed, the abdomen is darkly pigmented, the genitalia are malformed, and very low levels of yolk protein are expressed (Baker and Ridge 1980; Bownes and Nöthiger 1981). Besides these activities, analysis of *dsx* mutant animals indicates that the gene also functions in neurogenesis and behavior (Taylor and Truman 1992; Taylor *et al.* 1994; Villella and Hall 1996). The dsx^{Dom} alleles block the female-specific splicing of the *dsx* pre-mRNAs and constitutively express the male version of the Dsx protein (Nagoshi and Baker 1990). These alleles have no phenotypic effects in males, but disrupt normal sexual differentiation in females. XX flies carrying one copy of dsx^{Dom} ($dsx^{Dom}/+$) are intersexual but tend to exhibit more male-specific characteristics than female ones (Baker and Wolfner 1988). Replacing the wild-type copy of *dsx* with a null allele transforms dsx^{Dom} females into sterile pseudomales. Analysis of these gain-of-function alleles and of a Dsx^{M} transgene indicates that the Dsx^{M} protein also promotes sex-comb formation (Jursnich and Burtis 1993). Much less is understood about the specific functions of Dsx^{F} and no constitutive dsx^{F} alleles exist.

To better understand the activities of the female Dsx protein (Dsx^{F}), we generated an artificial "gain-of-function dsx^{F}" allele by ectopically expressing a *dsx* female cDNA under the control of the constitutive *hsp83* promoter. We have examined the effects of this artificial gain-of-function allele on sexual morphology, gene expression, and behavior in males (XY) wild type or mutant for the endogenous *dsx* gene. This analysis reveals that female and male sex-specific genes or pathways respond differently to Dsx^{F} and Dsx^{M}. For example, Dsx^{F} can induce the expression of female-specific genes such as the *yolk proteins* or those involved in the production of female pheromones even in the presence of Dsx^{M}. However, in other aspects of sexual differentiation such as cuticular development and the synthesis of a male-specific pheromone, Dsx^{M} either competes with Dsx^{F} on an equal footing or is dominant to Dsx^{F}. Though *hsp83-*dsx^{F}*;*dsx^{+} males are fertile and readily mate with females, they also form courtship chains and elicit high levels of courtship from wild-type males. Much more severe behavioral abnormalities are evident when the transgenic males lack *dsx*. Like females, these animals will copulate with wild-type males. Finally, we examined the role of the *intersex* (*ix*) gene. Previous studies have shown that females mutant in *ix* have an intersexual phenotype that closely resembles that observed in *dsx* mutants. However, unlike *dsx*, *ix* mutations have no phenotypic effects in males (Baker and Ridge 1980). To date, it has been difficult to determine whether the *ix* gene is under the control of the *Sxl* → *tra/tra-2* splicing cascade and expressed only in females, or whether it is constitutively expressed, like *tra-2*, in both sexes. By examining Dsx^{F} function in ix^{-} males, we discovered that *ix* is required for the feminization activities of Dsx^{F}. Our findings show that *ix* is not directly regulated by the *Sxl* → *tra/tra-2* splicing cascade and is most likely constitutively expressed in both sexes.

MATERIALS AND METHODS

Drosophila stock and plasmid constructions: Flies were grown on standard medium (Cline 1978) and raised at 25° unless otherwise indicated. The *doublesex* stocks used were w^{1}/*Bs;* dsx^{1}/*TM3 Ser*, the w^{1}/*Bs;* dsx^{43}/*TM3 Ser* as the dsx^{Df} allele, and the dsx^{Dom} allele used was the w^{1}/*Bs;* dsx^{Swe}/*TM3 Ser*. The *intersex* stocks used were w^{1}*;* pr^{1} cn^{1} ix^{1}/*SM5* and w^{1}*; Df(2R) enB* b^{1} pr^{1}/*CYO* as the ix^{Df} allele. Both *ix* stocks were obtained from the Bloomington Stock Center. The dsx^{F} cDNA was kindly provided by K. Burtis. To generate the *hsp83-*dsx^{F} transgene, an *Eco*RI/*Cla*I fragment containing the dsx^{F} cDNA was cloned behind the *hsp83* promoter in a *P*-element vector containing

the *miniwhite* gene pHS83Capser (Horabin and Schedl 1993). The P[*hsp83-dsx*^{F}] transgene characterized in detail here was arbitrarily designated line 26B. This transgene is located on the X chromosome and is homozygous viable. Two other *hsp83-dsx*^{F} transgenic lines also characterized, but in less detail, are 44A and 50B. Both of these lines are located on the second chromosome and are homozygous lethal. The *hsp83-tra*^{F} transgene was cloned in a similar manner using the *tra*^{F} cDNA (J. Waterbury, J. I. Horabin, D. Bopp and P. Schedl, unpublished results). To express the *dsxF* cDNA in a *yellow* reporter vector, the *yellow* gene (gift of Y. Hiromi) was cloned into Carnegie 4 (Pirrotta 1988) to create pYellC4. The *dsxF* cDNA was cloned behind the *hsp83* promoter in pHS83BS and the *Kpn*I/*Sma*I fragment containing hsp83-dsx^F was cloned into the *Sal*I site of pYellC4. The *o* reporter transgenes were reestablished using the pPW893 construct provided by P. Wensink. pPW893 contains the *o* element repeated four times upstream of *hsp70-lacZ* (An and Wensink 1995a).

Sample preparation and photography: Adult forelegs from flies raised at 25° were removed by dissection and fixed in a mixture of EtOH and glycerol (3:1). Samples were mounted in Hoyer's under a coverslip and heated to 60° for 1–2 hr. Photographs were taken using a Nikon camera mounted on a Nikon Microphot-SA at ×200–400 magnification. Adult abdomens were photographed using a Nikon SMZ-2T stereomicroscope at ×35–50 magnification.

β-Galactosidase activity and protein analysis: Flies were dissected in PBS, fixed in 3% glutaraldehyde, and stained for β-galactosidase activity overnight at room temperature according to the staining protocol of Bellen *et al.* (1989). Western analysis for β-galactosidase protein was performed using anti-β-galactosidase antibody supplied by Promega (Madison, WI) and antitubulin antibody supplied by Sigma (St. Louis).

Northerns: Total RNA was prepared according to the method described in Bopp *et al.* (1993). Northerns were blotted onto Zeta probe membrane (Bio-Rad, Richmond, CA). Plasmid containing *yp-1* cDNA (pYP1 of Hung *et al.* 1982) was provided by K. Burtis and used as a probe for *yolk protein* expression analysis. Blots were probed with ^{32}P-labeled *yp-1* cDNA or with ^{32}P-labeled *rp49* cDNA as a loading control. Blots were imaged on a Molecular Dynamics (Sunnyvale, CA) Phosphoimager and quantitated using Image Quant.

Behavior assays: Flies were collected after eclosion at 25° and kept in isolation for 1–3 days for virgin females and 4–6 days for males. To measure behavior, individual males were placed with a second fly in a small Plexiglas chamber (1 cm × 4 mm). Behavior was videotaped and measured by observation. Courtship index (CI) was measured for each test male and represents the percentage of time a particular fly performed courtship within a given period of time or until mating. Courtship behaviors are defined here as any of the following behaviors: following, orientation, tapping, wing extension and vibration, abdomen curling, and attempted copulation. Line-crossing assays were performed as described in Finley *et al.* (1997). In brief, the number of times a mating pair crossed an arbitrary line drawn across the diameter of the chamber during copulation was recorded. Calculations were performed using Microsoft Excel and are shown as the average ± standard error mean (SEM). *P* values were done using ANOVA one-way analysis on Microsoft Excel.

Pheromone analysis: Flies were collected after eclosion at 29°, aged for 4–5 days, frozen and stored at −70°. Extractions were done in *n*-hexane using 3 or 10 flies per extraction (Tompkins and McRobert 1989). Extracts were analyzed by gas chromatography/mass spectrometry on a 12-m HP-1 silicone capillary column programmed from 180° (2 min) to 295° at 5°/min in a HP 5890 gas chromatograph/HP 5971 mass spectrometer. The hydrocarbon peaks were quantified by total ion current comparisons to an internal standard. Saturated and branched hydrocarbons were identified by their characteristic mass spectra. Unsaturated hydrocarbons were characterized from mass spectra of their dimethyl disulfide derivatives (Carlson *et al.* 1989).

RESULTS

***Hsp83-dsx*^{F} transgene can rescue *dsx* females:** To learn more about the biological activities of Dsx^F, we generated a *P*-element construct with a *dsx* female cDNA placed under the control of the *hsp83* promoter. The *hsp83* promoter is constitutively active even at low temperatures and expression can be increased by shifting to higher temperatures. Nine independent *mini-white-hsp83-dsx*^{F} transgenic lines were recovered. To test the activities of the different transgenic lines, we introduced each into females lacking endogenous *dsx* (*w*^{1};*dsx*^{1}/*dsx*^{Df}). From an analysis of the phenotypes exhibited by the rescued females, we selected three lines (26B, 44A, and 50B) that had the greatest rescuing activity for further study, and in the following discussion, we describe one of these lines, *P[dsx*^{F}*26B]*. Similar results were obtained for the other two lines.

In single copy, the *hsp83-dsx*^{F}*26B* transgene was sufficient to restore some aspects of feminization to *dsx*$^-$ females at 18° and 25°, and all aspects at 29°. No effects on female viability were observed at 25° (n = 681) or 29° (n = 576) in the presence of endogenous *dsx* (*w*^{1},*P[dsx*^{F}*26B]*/*w*^{1};+/+ compared to *w*^{1};+/+). Subtle effects on viability (6–8%) were observed at 25° (n = 136) and 29° (n = 174) in the absence of *dsx* (*w*^{1},*P[dsx*^{F}*26B]*/*w*^{1};*dsx*^{1}/*Df* compared to *w*^{1};*dsx*^{1}/*Df*). At the lower temperatures, 18° to 25°, the genitalia were incompletely feminized, although clearly more feminized than the intersexual genitalia of XX animals that are *w*^{1};*dsx*^{1}/*dsx*^{Df} (data not shown). The male genital arch, normally found in *dsx*$^-$ females, was missing, although pigmentation of abdominal segments A6 and A7 was similar to that observed in *dsx*$^-$ females (Figure 1A). Whereas the ovaries of *dsx*$^-$/+ females have only incompletely formed, abnormal egg chambers, *w*^{1},*P[dsx*^{F}*26B]*/*w*^{1}; *dsx*^{1}/*Df* females had mature, overfilled ovaries containing multiple late-stage eggs (Figure 1A). The germline rescue of *dsx*$^-$ females was fully penetrant (n > 50). Hence, the transgene can provide sufficient wild-type Dsx^F function for normal oogenesis even at lower temperatures.

When raised at 29°, pigmentation and external genitalia of the *P[dsx*^{F}*26B];dsx*^{1}/*Df* animals resembled that of wild-type females (Figure 1, B and C). A single copy of the *dsx*^{F} transgene was able to rescue *dsx*$^-$ females to fertility at a frequency of 10–15% at 29° [13% rescue with 26B (n = 52)]. The remaining females were sterile and did not lay eggs. The cause of this egg-laying defect

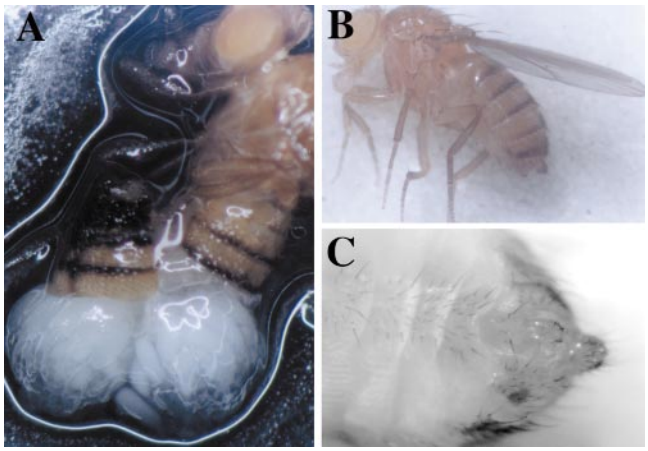

Figure 1.—Phenotypic analysis of *hsp83-dsx*^{F} transgene in *dsx*$^{-}$ females. (A) *w*^{1}, *P[83dsx*^{F}*26B]* / *w*^{1}*; dsx*^{1} / *Df* females raised at 25°. The abdomen was dissected and displayed open to show the mature ovaries filled with a differentiated germline of early and late stages of oogenesis. Incomplete rescue of the external abdominal pigmentation is also displayed. (B and C) *w*^{1}, *P[83dsx*^{F}*26B]* / *w*^{1}*; dsx*^{1} / *Df* females raised at 29°. (C) Abdominal view of the genitalia.

is unclear. The external genitalia and the internal somatic structures of the gonad of these sterile females closely resemble those of wild-type females (Figure 1C). Moreover, the ovaries of these sterile females are morphologically normal and filled with many late-stage eggs. It is possible that the egg-laying defect reflects a requirement for Dsx^{F} in the development of specific egg-laying muscles or innervation of these muscles. In either case, we presume that the *hsp83-dsx*^{F} transgene cannot provide sufficient Dsx^{F} activity at a critical point in development to ensure that feminization is complete in all animals. A similar defect in egg laying has also been observed in females mutant in *dissatisfaction* (*dsf*; Finley *et al.* 1997).

Expression of Dsx^{F} in males causes somatic feminization: To study the role of Dsx^{F} in sexual differentiation, we introduced the *hsp83-dsx*^{F} transgene into wild-type males. Males carrying one copy of the *hsp83-dsx*^{F} transgene were, in general, morphologically normal and fertile. (From here on, we will refer to *w*^{1}*,P[dsx*^{F}*26B];+ / +* males as *dsx*^{F} transgene males unless otherwise indicated.) At low temperatures, the only morphological alteration observed was the occasional appearance of sternite bristles with female-like characteristics in A6 (Figure 2A). When we increased the expression of *dsx*^{F} by raising the flies at 25° or 29°, the male-like genitalia were occasionally rotated. No effects on viability were observed at 25° (n = 758). However, when raised at 29°, the transgene caused a 34% reduction in male viability (n = 508).

Removing endogenous *dsx* (*i.e.*, Dsx^{M}) increased the ability of the *dsx*^{F} transgene to feminize XY flies. Removal of one copy of endogenous *dsx* from *dsx*^{F} transgene males (*w*^{1}, *P[dsx*^{F}*26B]* / *Bs; dsx*^{1} / +) had no effect on abdominal pigmentation, but did result in an increased

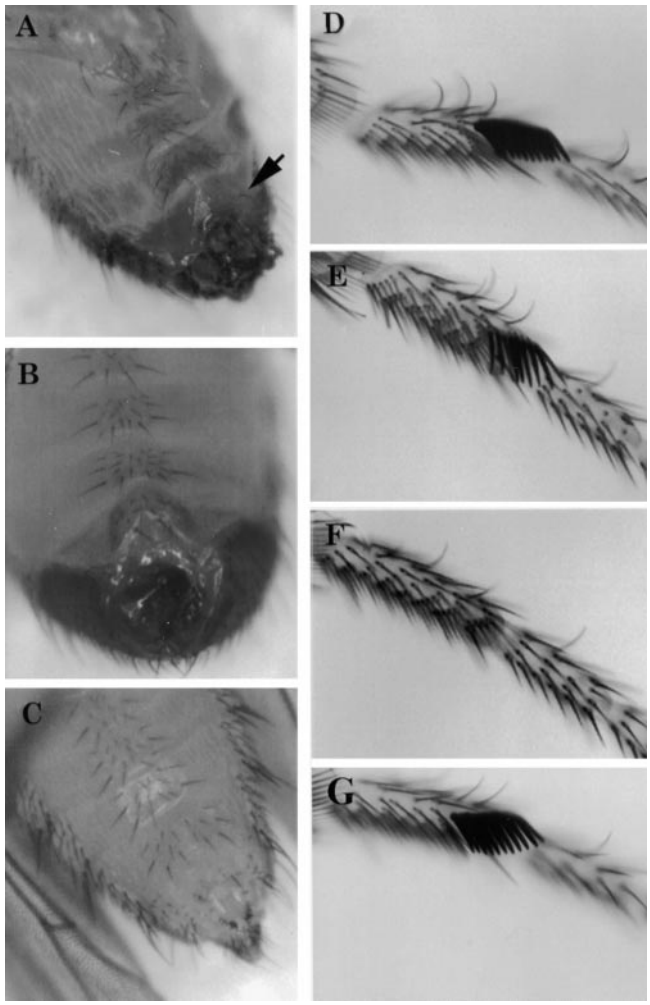

Figure 2.—Phenotypic analysis of sex combs and abdomens of dsx^{F} transgene males. (A–C) Photographs of the abdomens and genitalia from flies raised at 29°. (D–G) The basitarsus of the prothoracic leg showing sex combs from XY flies raised at 29°. (A and D) *w*^{1}, *P[83dsx*^{F}*26B]* / *Y;* + / +, (B and E) *w*^{1}, *P[83dsx*^{F}*26B]* / *B*^{s}*; dsx*^{1} / +, (C and F) *w*^{1}, *P[83dsx*^{F}*26B]* / *B*^{s}*; dsx*^{1} / *Df,* (G) *w*^{1} / *B*^{s}*;dsx*^{1} / *Df.* The arrow in A indicates bristle on A6. Hundreds of animals of each genotype were examined for genital defects. The phenotype shown in B of the *w*^{1}, *P[83dsx*^{F}*26B]* / *B*^{s}*; dsx*^{1} / + genitalia was fully penetrant with variable expressivity ranging from severely rotated genitalia to gross morphological defects. The phenotype shown in C of the *w*^{1}, *P[83dsx*^{F}*26B]* / *B*^{s}*; dsx*^{1} / *Df* genitalia was fully penetrant.

frequency of genital rotation and other gross abnormalities of the genitalia (Figure 2B). The number of bristles observed in A6 also increased (Figure 2B). Internally, however, these males still had male-specific accessory glands and the gonads developed as sperm-producing testes (data not shown).

Complete removal of endogenous *dsx* (*w*^{1}*, P[dsx*^{F}*26B]* / *Bs; dsx*^{1} / *Df*) transformed XY flies into pseudofemales when raised at 25° or 29°. At 25° (n = 140) and 29° (n = 144), the *hsp83-dsx*^{F} transgene reduced the viability of *w*^{1}*, P[dsx*^{F}*26B]* / *Bs; dsx*^{1} / *Df* males compared to *w*^{1} / *Bs; dsx*^{1} / *Df* males by 38–40%. Externally, these XY pseudofe-

males had female abdominal pigmentation and female genitalia, and lacked sex combs (see Figure 2C). External feminization was fully penetrant with invariant expressivity ($n > 100$). Internally, the transformation was incomplete. Although a uterus was present and male-specific structures like the accessory glands were absent, the germline and surrounding soma were underdeveloped. There were no distinct ovarioles or developing egg chambers; only amorphous germline/soma cell clusters (resembling early female sterile mutants, such as *bag-of-marbles*) were observed. This result differs from the germline rescue observed in w^1, *P[dsx^{F}26B]/Bs; dsx^1/Df* females and could reflect a difference in germline chromosomal composition combined with the absence of a complete sex determination signal from the soma (J. Waterbury, unpublished results).

The *dsx^F* transgene did not affect all aspects of sexual dimorphism. Consistent with previous studies that show that *dsx* mutations do not alter the body size of XX or XY animals (Cline 1984), pseudofemales that are w^1, *P[dsx^{F}26B]/Bs; dsx^1/Df* are no larger than wild-type males. *Hsp83-dsx^F* pseudofemales also have the male-specific abdominal muscles, the Muscles of Lawrence (data not shown). This result is not surprising because it has been demonstrated that development of this muscle is downstream of the *fruitless* pathway and independent of *dsx* (Taylor 1992).

Dsx^F acts as a negative regulator of sex comb formation: Using our constitutively expressed *dsx^F* transgene, we asked if Dsx^F could interfere with the formation of sex combs on the basitarsus of the foreleg in males. Females do not have sex combs but, instead, a traverse row of bristles. Males and females homozygous for loss-of-function *dsx* alleles have bristles that are not aligned as a traverse row and do not resemble sex comb teeth (Baker and Ridge 1980). Dsx^M has been shown to have a positive role in sex-comb formation; intermediate sex combs form when Dsx^M is expressed in females carrying a *dsx^{Dom}* allele (Baker and Ridge 1980) and ectopic sex combs form on all six legs in males or females when Dsx^M is expressed ubiquitously under *hsp70* control (Jursnich and Burtis 1993). Dsx^F, however, did not appear to affect sex-comb formation in otherwise wild-type males when *dsx^F* was ectopically expressed using an *actin-dsx^F* or an *hs70-dsx^F* transgene (Jursnich and Burtis 1993). These results led to the idea that Dsx^F does not play a role in the formation of sex combs.

Similar to previous results, a single copy of the *hsp83-dsx^F* transgene had no readily apparent effects on sex-comb formation in otherwise wild-type males (Figure 2D). To look more closely for a competitive balance between Dsx^F and Dsx^M on sex comb formation, endogenous copies of *dsx* were removed. By reducing the level of endogenous *dsx* (w^1, *P[dsx^{F}26B]/B^s; dsx^1/+*), Dsx^F was able to influence sex comb formation, resulting in phenotypically intersexual or intermediate sex combs with the teeth becoming more bristle-like (compare Figure 2, E and G). This was quantitated by comparing the number of sex-comb teeth in w^1/B^s; *dsx^1/+* and w^1, *P[dsx^{F}26B]/B^s;dsx^1/+* males. The average number of sex-comb-like bristles for nontransgene males was 10.03 ± 0.008 ($n = 39$) and for males with the transgene, 9.00 ± 0.55 ($n = 59$) ($p\ [X^2 \leq 31.55] = 6.23 \times 10^{-7}$). Removal of all endogenous *dsx* resulted in complete loss of sex combs and transformation to female bristles (Figure 2F). These results suggest that Dsx^F acts to negatively regulate sex comb formation in females and indicates that a competition exists between Dsx^F and Dsx^M when both protein forms are present, as suggested by Jursnich and Burtis (1993). This is the first example of a negative role for Dsx^F.

Dsx^F is a dominant activator of *yp* expression: To date, the only known direct target of Dsx binding is the fat body enhancer (FBE) that lies directly between the two yolk protein genes, *yp-1* and *yp-2*. Both forms of Dsx bind to the same sites within the FBE (Erdman and Burtis 1993; An and Wensink 1995b) with opposite regulatory effects on *yp-1* transcription: Dsx^F activates and Dsx^M represses (Burtis *et al.* 1991; Coschigano and Wensink 1993). *Yp-1* is expressed in both the fat body and the ovaries of females (Figure 3A, lanes 1 and 2); however, Dsx only regulates expression within the fat body (An and Wensink 1995a; Figure 3A, lanes 3 and 4). Dsx^F is not absolutely essential for *yp-1* expression in the fat body. In the complete absence of *dsx* activity, XX and XY animals express low levels of *yp-1* (Bownes and Nothiger 1981).

As shown in Figure 3, one copy of the *hsp83-dsx^F* transgene in otherwise wild-type males (*P[dsx^{F}26B]/Y; +/+*) was sufficient to activate *yp-1* expression in the fat body. This result confirms previous results using *actin-dsx^F* and *hs70-dsx^F*, demonstrating that Dsx^F acts to positively regulate *yp-1* expression in the fat body (Burtis and Baker 1989; Jursnich and Burtis 1993). The level of *yp-1* expression induced in transgene males was only ~2.5-fold less than that expressed in the fat body of wild-type females (see Figure 3, A and B).

According to the competition model for Dsx binding to the FBE and the phenotypic effects observed on sex combs described earlier, one would expect to see an increase in *yp-1* expression as the level of negatively competing Dsx^M is reduced. To test this, we varied the dose of endogenous *dsx* and measured the amount of *yp-1* mRNA transcript. As predicted, the levels of *yp-1* mRNA increased as endogenous *dsx* was reduced or completely removed (Figure 3B, compare lanes 4, 5, and 6). Although mRNA expression levels changed as a result of *dsx* gene dosage, Dsx is not the only factor responsible for *yp* gene regulation (see results below and discussion).

We also tested whether the *hsp83-dsx^F* transgene could drive expression of an *hsp70-lacZ* reporter construct through an upstream minimal fat body enhancer element, *o* (An and Wensink 1995a). The *o* enhancer con-

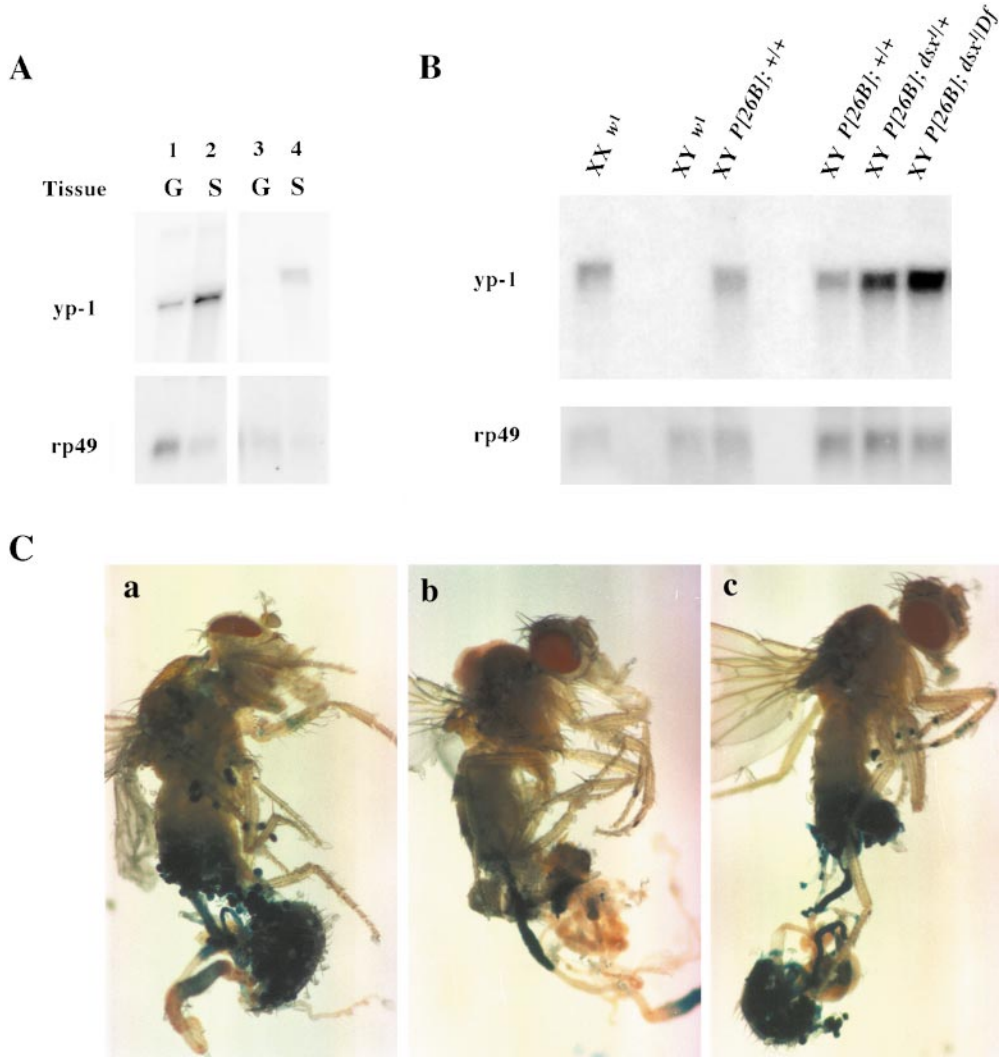

Figure 3.—Analysis of *yp-1* expression in dsx^F transgene males. (A) Total RNA was isolated from dissected gonadal (G) and somatic (S) tissues of w^1 control females (lanes 1 and 2) and w^1, $P[dsx^F26B]/Y$; +/+ males (lanes 3 and 4) and analyzed by Northern. (B) Total RNA was isolated from all samples indicated and analyzed by Northern. Lanes are numbered from left to right. Lane 1 represents total RNA from one-half of a single, whole female. All other lanes (lanes 2–7) represent total RNA from three flies each. $P[26B] = P[hsp^{83}dsx^F26B]$. Top panels in A and B were probed with ^{32}P-labeled *yp-1* cDNA and bottom panels were probed with ^{32}P-labeled *rp49* cDNA for comparison. Blots were imaged on a Molecular Dynamics Phosphoimager and quantitated using Image Quant. (C) Flies carrying the *o element:hsp70:lacZ* reporter were analyzed for β-galactosidase activity in the fat body tissues. (a) XX control female, (b) XY control male, and (c) w^1, $P[hsp^{83}$-$dsx^F26B]/Y$ male.

tains a Dsx protein-binding site, an overlapping aef1 transcription factor-binding site, and an overlapping, potential bZip protein-binding site (diagrammed in Figure 4A). Four tandem copies of the *o* element upstream of the LacZ reporter are sufficient for expression in wild-type females but not in wild-type males or XX;dsx^- flies (An and Wensink 1995a). As shown in Figure 3C, ectopic expression of Dsx^F in dsx^+ males induces *lac-Z* expression from the *o* element:*hsp70-lacZ* reporter. Thus, a reporter containing only the minimal Dsx enhancer responds like the endogenous *yp1* gene to the feminizing activities of the *hsp83-*dsx^F transgene.

Using the *o* element:*hsp70-lacZ* reporter, we examined β-gal protein expression levels in *hsp83-*dsx^F transgene males with varying levels of endogenous *dsx*. Shown in Figure 4B is the expression of β-gal protein in flies carrying the reporter. Lanes 1 and 2 demonstrate the sex specificity of the reporter in a wild-type background without the *hsp83-*dsx^F transgene. Subsequent lanes show β-gal expression in *hsp83-*dsx^F transgene male sibling pairs from three independent crosses either wild type and heterozygous (lanes 3 and 4) or heterozygous and homozygous (lanes 5 and 6, 7 and 8) for the *dsx* locus. In each pair, the level of β-gal increased as the dose of the endogenous *dsx* gene decreased. Similar results were obtained when β-gal mRNA expression was examined (data not shown).

Activation of *yp-1* expression via Dsx^F is *ix* dependent: It has been hypothesized that *ix* acts in parallel with or downstream of *dsx* in females (Chase and Baker 1995). This hypothesis is based principally on the similarity of *dsx* and *ix* mutant phenotypes in females. Females homozygous mutant for *ix* have an intersexual phenotype that closely resembles that of *dsx* mutant animals. Additionally, expression of *yp-1* mRNA is greatly reduced in ix^- females (Figure 5, lane 7) and the level of mRNA is comparable to that seen in dsx^- females. On the other hand, unlike dsx^- males, males homozygous for ix^- have no observable phenotype and do not express detectable levels of *yp-1* mRNA (Figure 5, lane 3). The latter result indicates that Dsx^M can repress *yp-1* transcription in the absence of Ix protein.

Given that Dsx^F can induce *yp-1* expression in males, we looked to see if this induction is dependent upon

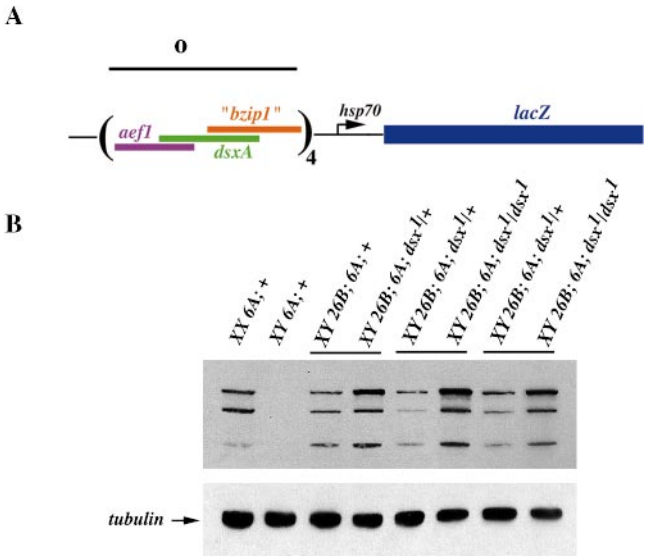

Figure 4.—Dsx^F regulation through the *o* element enhancer is dependent on *dsx* gene dosage. (A) Diagram of the *o* element minimal enhancer reporter gene. The *o* element is repeated in four copies upstream of the *hs70* promoter and the *lacZ* gene. For gene construction, refer to An and Wensink (1995a). The *o* element contains three overlapping sites: *aef1*, *dsxA*, and a potential bzip binding site, "*bzip1*." (B) Western analysis of β-galactosidase protein as a function of *dsx* gene dosage. All flies in this assay are *ry*$^-$ and carry the *P*[*o element:hsp70:lacZ*] designated as *6A*. Relevant genotypes are shown above each lane. The *26B* designation is described in the legend to Figure 2. The single lines shown above lane pairs designate those lanes as sibling pairs from independent crosses. Each lane contains the equivalent of one-fourth of a fly. The blot was probed with an anti-β-galactosidase antibody (top) and subsequently with an antitubulin antibody (bottom). The arrows indicate β-galactosidase and tubulin proteins. The other bands in the top panel are breakdown products and are not detected in control males (XY 6A;+).

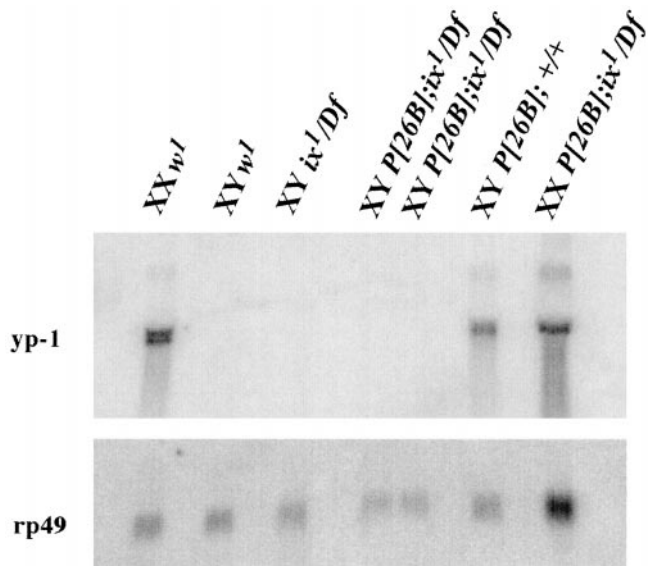

Figure 5.—Northern analysis of *yp-1* expression in *dsx*^{F} transgene males mutant for *ix*. Total RNA was isolated from all samples indicated. (Lanes are numbered from left to right) Lane 1 (*w*^{1} control females) represents total RNA from one-half of a single female and lane 7 (*w*^{1}, *P[dsx*^{F}*26B]/w*^{1}*; ix*^{1}*/ix*^{Df}) represents total RNA from two whole females. All other lanes (lanes 2–6) represent total RNA from three flies each. Lanes 4 and 5 represent two independent crosses, *P[26B] = P[hsp83dsxF26B]*. (Top) Probed with ^{32}P-labeled *yp-1* cDNA; (bottom) probed with ^{32}P-labeled *rp49* cDNA for comparison. All flies were raised at 25°. Blots were imaged on a Molecular Dynamics Phosphoimager and quantitated using Image Quant.

ix. Males carrying the *dsx*^{F} transgene, but homozygous for *ix*$^-$ and wild type for *dsx* (*w*^{1},*P[dsx*^{F}*26B]/Y; ix*^{1}*/ix*^{Df}*;* +/+), are phenotypically wild type and fertile. However, without *ix*, Dsx^F was no longer able to induce expression of *yp-1* (Figure 5; compare lanes 4 and 5 with 6). Similarly, in males, induction of LacZ expression from the *o* element *hsp70-lacZ* reporter by the *dsx*^{F} transgene was also dependent upon the *ix* gene (data not shown). These findings suggest that Dsx^F and Ix function synergistically to activate full transcription of *yp-1* in the fat body. They also indicate that Ix is either constitutively expressed in males or is under the direct control of the Dsx^F protein.

Behavior: The courtship behavior of wild-type Drosophila males has been well characterized and involves a series of choreographed routines. It begins with an orientation of the male toward the female, followed by wing extension and vibration to produce stimulatory songs, tapping, licking of the female genitalia, mounting, abdomen curling, and finally copulation (see review by Hall 1994). The genetic regulatory circuits controlling these different sexual behaviors appear to be more complicated than those involved in directing the differentiation of male- (or female)-specific adult cuticular structures. At least three genes are known to contribute to sexual behavior: *dsx*, *dissatisfaction* (*dsf*), and *fruitless* (*fru*) (reviewed by Hall 1994; Taylor *et al.* 1994; Finley *et al.* 1997). Mutations in all three genes alter male sexual behavior and/or neurogenesis, while female behavior and/or neuronal development are affected only by *dsx* and *dsf* mutations. As with *dsx*, *fru* is alternatively spliced in females by the *Sxl* → *tra*/*tra-2* splicing cascade and thus *fru* is independent of *dsx* (Ito *et al.* 1996; Ryner *et al.* 1996). Although genetic studies have suggested that *dsf* is also under the control of the *Sxl* → *tra*/*tra-2* splicing cascade (Finley *et al.* 1997), recent cloning and additional analysis of *dsf* have suggested that it represents a *tra*/*tra-2* independent pathway (Finley *et al.* 1998).

dsx$^-$ males have a lower measured courtship index toward females than wild-type males, exhibiting a reduced frequency of wing extension and song singing, and are defective in the production of the sine song (Villella and Hall 1996). *dsf*$^-$ males, on the other hand, actively court with nearly normal courtship routines; however, they fail to discriminate between the sexes and court males with the same avidity as females

TABLE 1

Courtship activity of transgene males toward wild-type females

Genotype[a]	*n*	CI[b]	Duration of copulation[c]
XY OreR	18	56.6 ± 5.7	21.1 ± 0.6 (16)
w^1, *P[dsx^{F}26B]/Y*	20	51.4 ± 4.6	17.6 ± 0.8 (19)
w^1/B^s; *P[83tra^{F}5.4]*	17	1.1 ± 0.4	—
w^1/B^s; $dsx^1/+$	18	22.6 ± 3.0	—
w^1, *P[dsx^{F}26B]/B^s*; $dsx^1/+$	21	9.5 ± 2.5	—
w^1, *P[dsx^{F}26BB^s]*; dsx^1/Df	16	0.14 ± 0.1	—
w^1/B^s; dsx^1/Df	10	11.8 ± 5.2	—

[a] All are of XY chromosomal composition. All transgenes (*P[dsxF*]* and *P[traF*]*) are in single copy.

[b] CI is courtship index ± SEM. See text for *P*-value comparisons. See materials and methods for definition of CI.

[c] Represents an average time (minutes). *n* is indicated in parentheses.

(Finley *et al.* 1997). They are also slow to copulate, due to defects in abdominal neuronal development that affect abdominal curling. Mutations in *fru* cause a number of defects in male courtship (reviewed by Hall 1994). *fru*$^-$ males court with greatly reduced vigor compared to wild-type males, and the later courtship routines, such as singing and copulation, are abnormal or missing. Finally, *fru*$^-$ males court males and females with equal avidity.

We asked whether the *dsx*^{F} transgene had any effects on male courtship behavior. As a (partial) control for these experiments, we also examined the effect of another transgene, *hsp83-tra*^{F}, on male courtship behavior. This transgene expresses female Tra protein, and together with Tra-2, should direct the female-specific expression not only of *dsx*, but also of *fru*. The *hsp83-tra*^{F} transgene is expected to more strongly feminize XY animals than *hsp83-dsx*^{F}; however, the feminization of XY animals by the *hsp83-tra*^{F} is not complete in all tissues, and male-specific structures, such as the Muscle of Lawrence, are still observed (data not shown). When *hsp83-tra*^{F} pseudofemales were placed in individual chambers with another male or female, they showed little interest in courting. When they did court, they did not discriminate between males and females and only very early courtship routines were observed, such as orientation, tapping, and brief wing vibration.

As mentioned earlier, *dsx*^{F} transgene males are fertile and can and will mate with females. The measured courtship index of w^1,*P[dsx^{F}26B]/Y*; +/+ males, shown in Table 1, demonstrates that they court virgin females with as much interest as wild-type males ($P = 0.49$). The duration of copulation of *dsx*^{F} transgene males was also measured and there is a significant, slight reduction in the time of copulation compared to wild-type males ($P = 0.0012$; Table 1).

We also tested whether the *dsx*^{F} transgene males would discriminate between females and males. Unlike *dsf*$^-$, *fru*$^-$, or *hsp83-tra*^{F} males, *dsx*^{F} transgene males did not court wild-type males (data not shown). However, we did find that wild-type males courted transgene males and that transgene males courted each other with significant courtship indices (Table 2 and data not shown). *Hsp83-tra*^{F} pseudofemales also elicited high levels of courtship from wild-type males (Table 2). It has been shown previously that misexpression of the *white* gene can cause abnormal courtship behavior in males (Zhang and Odenwald 1995; Hing and Carlson 1996). Because *mini-white* is the marker used for our *hsp83-dsx*^{F} construct, we were concerned that the behavioral defects we observed might arise from *mini white* expression. To control for this possibility, we generated a new version of the *hsp83-dsx*^{F} transgene using *yellow* as the transformation marker. As shown in Table 2, we found that *P[hsp83-dsx^F-yellow];+/+* males were as attractive to wild-type males and to each other as *P[hsp83-dsx^{F}mini-white];+/+* males. This finding argues that the attractiveness of transgenic males to wild-type males and to each other is due to ectopic expression of Dsx^F rather than misexpression of *mini-white*. Given that *dsx*^{F} transgene males do not court wild-type males, the observed behavioral abnormalities are unlikely to be due to an inability to discriminate between the sexes. Rather we suspect that the *dsx*^{F} transgene males produce female attractants that are responsible for eliciting courtship behaviors by other males. We address this possibility further below.

While the *dsx*^{F} transgene had no apparent effect on the courtship behavior of (otherwise) wild-type males toward females, courtship behavior could be altered by reducing the dose of *dsx* gene (Table 1). To distinguish XY *dsx*^{F} males from XX females in this experiment, we marked the XY animals with the Y chromosome-linked

TABLE 2

Courtship elicited by transgene males

Genotype of elicitor[a]	*n*	CI of OreR males[b]
XY OreR	20	0.3 ± 0.1
w^1, *P[83dsx^{F}26B-mw]/Y*	20	78.3 ± 3.1
w^1, *P[83dsx^F 12C-y]/Y*	13	81.0 ± 4.0
w^1/B^s; *P[83tra^{F}5.4]*	15	87.2 ± 3.5
w^1, *P[83dsx^{F}26B]/B^s*; dsx^1/Df	11	89.2 ± 2.2

mw, miniwhite; *y, yellow*.

[a] All are of XY chromosomal composition. All transgenes (*P[dsxF*]*, *P[dfy*]*, and *P[traF*]*) are in single copy.

[b] CI is courtship index ± SEM. See materials and methods for CI definition. ANOVA one-way comparisons were performed. Single pairwise comparison between *OreR* and *P[dsx^{F}26B-mw]* gives $P = 9.0 \times 10^{-25}$. Single pairwise comparisons between *P[dsx^{F}26B-mw]* and *P[dsx^{F}12C-y]*, *P[tra^{F}5.4]*, and *P[dsx^{F}26B-mw];dsx^1/Df* result in respective *P*-values of $P = 0.60$, $P = 0.07$, $P = 0.025$.

eye marker B^s. The defect caused by B^s has been shown to cause a twofold reduction in male courtship (Villella and Hall 1996). In this genetic background, the courtship index of $w^1/B^s;dsx^1/dsx^+$ control males is slightly less than half that of wild-type males (or w^1, $P[dsx^F26B]/Y;+/+$ males; $P = 9.0 \times 10^{-6}$). Because previous studies by McRobert and Tompkins (l985) indicate that males heterozygous for dsx^- court as wild-type males do, this twofold reduction in the courtship index is likely due to the impaired visual system of the B^s animals. We examined the courtship of transgenic XY animals either heterozygous or homozygous for dsx^-. As shown in Table 1, heterozygous transgenic males (w^1, $P[dsx^F26B]/B^s;\ dsx^1/+$), courted less frequently and less aggressively than the $w^1/B^s;dsx^1/+$ controls, and when they did court, it was not sustained for long periods of time ($P = 0.0021$). Even more severe defects in courtship behavior were evident for dsx^F pseudofemales (w^1, $P[dsx^F26B]/B^s;dsx^1/dsx^{Df}$; $P = 0.0083$ when compared to CI of dsx^1/dsx^{Df}). They showed little interest in females and performed only early mating behaviors (orientation, tapping, wing extension and vibration). The courtship index of these pseudofemales was comparable, although significantly less than that of the $hsp83\text{-}tra^F$ transgene males ($P = 0.013$).

Because the dsx^F pseudofemales exhibited reduced male courtship behavior, we asked whether these pseudofemales would respond like wild-type females to courtship by wild-type males. While dsx^F pseudofemales actively rejected courting wild-type males, they did allow themselves to be mated. Unlike wild-type females, however, the dsx^F pseudofemales continued to move around the chamber during copulation and flick their wings in an apparent attempt to dislodge the male. This difference in activity during copulation is evident in the relative frequency of line crossing by wild-type females and dsx^F pseudofemales ($P = 6.31 \times 10^{-5}$; see Table 3 and materials and methods). In addition, as shown in Table 3, dsx^F pseudofemales took two- to threefold longer to mate than wild-type females ($P = 8.0 \times 10^{-4}$). Although never observed by us, $P[hsp83tra^F]$ XY pseudofemales will also allow themselves to be mated by wild-type males (B. J. Taylor, personal communication).

DsxF acts as a dominant regulator of female pheromones: A plausible explanation for the high levels of courtship elicited from wild-type males by dsx^F transgene males is that ectopic expression of Dsx^F protein induces the expression of female pheromones. Pheromones are produced by oenocytes located directly beneath the adult abdominal cuticle and consist of several long chain hydrocarbons (Antony and Jallon 1982; Ferveur 1997). Females and males each generate their own characteristic aphrodisiac and antiaphrodisiac pheromones. Two long-chained compounds characterized as male attractants, 7,11-heptacosadiene (7,11-27:2 or 7,11-HCD) and 7,11-nonacosadiene (7,11-29:2 or 7,11-NCD), are produced by females (Antony *et al.* 1985). Females also produce two minor compounds 27:0 and 7-27:1. Males lack these female-specific compounds and instead produce compounds thought to be antiaphrodisiacs, such as 5-tricosene (5-23:1 or 5-T) and 7-tricosene (7-23:1 or 7-T; Jallon 1984; Scott 1986, 1996; Cobb and Jallon 1990; Cobb and Ferveur 1996a). Although 5-T is only present in rather small quantities in wild-type males, it has significant inhibitory effects on male courtship (Ferveur and Sureau 1996; Ferveur 1997). Wild-type females produce only trace amounts of 5-T. As can be seen in Figure 5B and Table 4, the antiaphrodisiac 7-T is present in both sexes; however, males produce much higher levels than females.

dsx previously has been shown to have a role in the production of these pheromones (McRobert and Tompkins 1985; Jallon *et al.* 1988) but that role has not been fully defined. To a first approximation, the pheromone profile of homozygous dsx^- females resembles that of wild-type males. *dsx* mutant females have little or no 7,11-NCD or 7,11-HCD, and instead produce reduced levels of the two minor female-specific hydrocarbons 27:0 and 7-27:1 and high levels of the male hydrocarbons 7-T and 5-T (Table 4, Figure 6, A and B and Jallon *et al.* 1988). The pheromone profile of dsx^- XY animals is similar to that of wild-type males in that the levels of 7-T and 5-T remain high; however, unlike wild-type males, dsx^- males have small but detectable amounts of the female aphrodisiac 7,11-NCD, and produce the two minor female-specific hydrocarbons, 27:0 and 7-27:1, at levels comparable to dsx^- females (Table 4).

We found that introduction of one copy of the dsx^F transgene into otherwise wild-type males is sufficient to dramatically alter the pheromone profile of these XY animals. The results are most straightforward for the two major female-specific aphrodisiacs, though similar changes are observed for the minor female-specific hydrocarbons, 27:0 and 7-27:1. In contrast to wild-type males, dsx^F transgene males produce significant amounts of the female-specific dienes, 7-HCD (168.5 ng/fly) and 7-NCD (42.0 ng/fly; Table 4, Figure 6A). When one copy of the endogenous *dsx* gene is removed, the levels of 7,11-NCD increase while the amount of 7,11-HCD drops slightly (Table 4, Figure 6A). Essentially the same female pheromone profile is observed when both endogenous alleles are removed. These results suggest that Dsx^F has a positive effect on the production of the female-characteristic compounds 7,11-HCD and 7,11-NCD and would account for the lack of either diene in wild-type males and dsx^- females.

Production of male-characteristic pheromones was also altered in dsx^F transgene males. One copy of the dsx^F transgene was sufficient to reduce the levels of the potent male antiaphrodisiac 5-T to trace amounts (10.0 ng/fly), a level similar to that detected in control females (10.0 ng/fly). This is much less than that found in XX and XY *dsx* mutants (31.5 and 52.0 ng/fly, respectively; see Table 4). As observed for the female-specific

TABLE 3

Behavior in response to OreR males

Genotype[a]	Time to mate[b]	Line crossing[c]
XX OreR	5.1 ± 0.7 (29)	6.6 ± 1.1 (29)
XYw[1], P[dsxF26B]/B[s]; dsx[1]/Df	13.3 ± 2.6 (18)	21.4 ± 4.0 (14)

n is indicated in parentheses.
[a] The *dsxF26B* transgene is in single copy.
[b] The time expired (in minutes) before copulation occurred ± SEM.
[c] The number of times during copulation the female crossed an arbitrary line.

compounds 7,11-HCD and 7,11-NCD, this effect on 5-T production is largely independent of endogenous *dsx*. Together with the observation that relatively high levels of 5-T were found in XX and XY *dsx* mutants, these results suggest that reduction of 5-T synthesis caused by Dsx^F cannot be overcome by Dsx^M.

Production of the antiaphrodisiac 7-T was also reduced by Dsx^F. The amount of 7-T decreased nearly 10-fold from 836.0 ng/fly in wild-type males to 93.0 ng/fly in dsx^F transgene males. As shown in Table 4 and Figure 6A, this amount is less than that detected in XX or XY *dsx* mutants (411.5 and 588.5 ng/fly, respectively) and close to that measured in wild-type females (103.0 ng/fly).

Three lines of evidence argue that the changes observed in hydrocarbon profiles are a consequence of dsx^F expression. First, similar results were obtained in all dsx^F transgenic lines examined. Second, males transgenic for a control *mini-white* construct, *P[hsp83-lacZ-mw]*, have a male-characteristic hydrocarbon profile (data not shown). Finally, we examined the pheromone profile of males transgenic for *hsp83-tra*^{F}. As expected from studies on *UAS-tra* males by Ferveur *et al.* (1997), the pheromone profile of *hsp83-tra*^{F} males resembles that of wild-type females. However, we found that tra^F feminizes the pheromone profile solely by directing expression of *dsx* in the female mode because *P[hsp83-tra*^{F}*]*; dsx^{Swe}/dsx^{Df} males have a male-characteristic profile and do not produce either of the female-specific dienes, 7,11-HCD or 7,11-NCD (data not shown).

In addition to the long-chained hydrocarbons synthesized by oenocytes under the adult cuticle, another male-specific compound, *cis*-vaccenyl acetate (cVA), is produced by the ejaculatory bulb in males and transferred to females during mating (Butterworth 1969). XX and XY *dsx* mutants both produce cVA (Table 4; Jallon *et al.* 1988). Unlike production of 5-T and 7-T, production of cVA does not seem to be strongly affected by Dsx^F in the presence of Dsx^M, although quantitated amounts of cVA do decrease from 160.0 ng/fly in control males to 45.0 and 107.0 ng/fly in dsx^F;+/+ and dsx^F; $dsx^1/+$ males, respectively (Table 4). However, when all endogenous *dsx* is removed, no cVA can be detected in XY flies carrying the transgene. Because XX and XY

TABLE 4

Chemical analysis of dsx^F transgene males

	Cuticular hydrocarbons						
Genotype	7,11-NCD	27:0	7-27:1	7,11-HCD	5-T	7-T	cVA
XXw^1	157.0 (8.5)	26.5 (1.4)	54.5 (2.9)	366.5 (19.9)	10.0 (0.5)	103.0 (5.6)	—
XY w^1	—	—	—	—	73.0 (3.9)	836.0 (44.8)	160.0
XXdsx^1/dsx^{Df}	9.0 (0.7)	7.5 (0.6)	17.5 (1.3)	—	31.5 (2.3)	411.5 (31.3)	140.0
XYdsx^1/dsx^{Df}	7.0 (0.4)	11.0 (0.7)	11.5 (0.7)	—	52.0 (3.2)	588.5 (36.1)	125.0
XYdsx^F26B; +/+	42.0 (4.4)	33.5 (3.6)	28.0 (2.8)	168.5 (17.9)	10.0 (1.0)	93.0 (9.8)	45.0
XYdsx^F26B; $dsx^{1/+}$	114.0 (10.3)	90.0 (8.1)	49.0 (4.4)	137.0 (12.4)	—	22.5 (2.0)	107.0
XYdsx^F26B; dsx^1/dsx^{Df}	219.5 (17.1)	128.5 (10.0)	78.5 (6.1)	193.0 (15.1)	—	17.0 (1.3)	—
XXix^1/ix^{Df}	—	29.4 (1.6)	206.7 (11.0)	—	26.7 (1.4)	295.7 (15.7)	—
XYix^1/ix^{Df}	—	16.5 (1.0)	—	—	50.1 (3.1)	727.6 (44.0)	111.7
XYdsx^F26B; ix^1/ix^{Df}	—	22.2 (1.4)	37.2 (2.5)	—	43.3 (2.8)	348.2 (23.0)	162.2

Amounts are presented as nanograms/fly. Values in parentheses represent the percentage of total cuticular hydrocarbons. All animals are in a w^1 background.

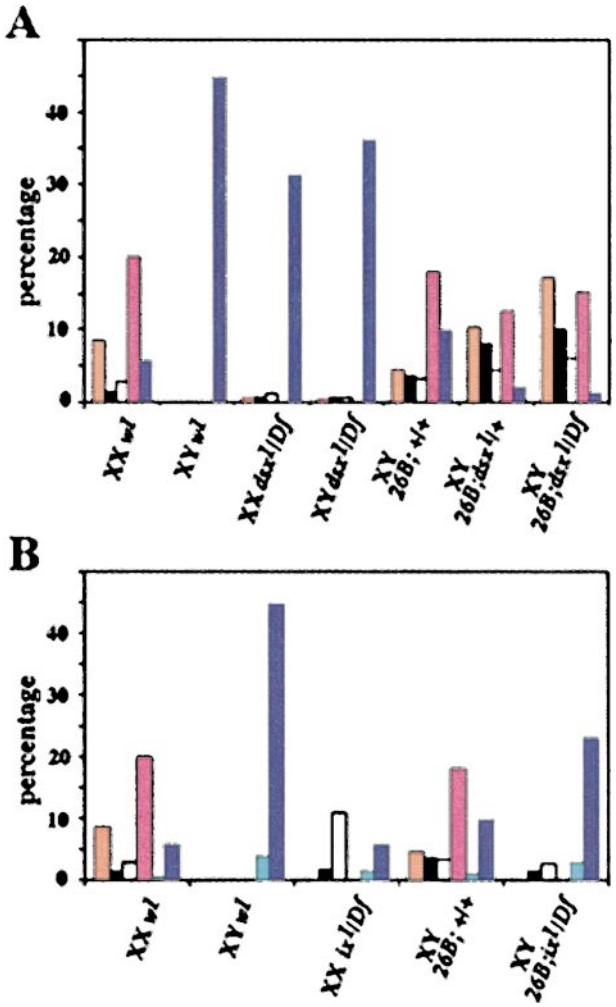

Figure 6.—Hydrocarbon profiles can be altered by expression of dsx^F. (A) Profiles of female-specific hydrocarbons and 7-tricosene production. Genotypes for the profile are indicated below. (B) Female hydrocarbon production is dependent on *ix*. Genotypes for each profile are indicated. All values represent a percentage of the total cuticular hydrocarbons. Compounds are indicated by the following colors: light pink, 7,11-NCD; black, 27:0; white, 7-27:1; dark pink, 7,11-HCD; dark blue, 7-T; light blue, 5-T.

dsx^1 / *Df* flies produce cVA, it is possible that cVA production is negatively regulated by Dsx^F but only efficiently in the complete absence of Dsx^M.

***Intersex* mutations block expression of female pheromones:** Given that *ix* appears to have a role in yolk protein production in dsx^F transgene males, we looked to see if there is a similar dependence on *ix* for pheromone production. Males mutant for *ix* had a hydrocarbon profile similar to wild-type males and dsx^- males, indicating that no role for *ix* can be assigned in males under these conditions (Table 4). This result is in agreement with all previous results regarding the lack of phenotypic effects of *ix* in males. Females mutant for *ix*, on the other hand, produced a hydrocarbon profile very different from that of wild-type females (Table 4, Figure 6B). Similar to the profile of dsx^- females, 7,11-HCD and 7,11-NCD were not detectable in ix^- females, suggesting that *ix* is required for the production of these two female-specific compounds. This result is in contrast to that described earlier for *yp-1* expression in the fat body where neither *ix* nor *dsx* alone is required for basal *yp-1* expression, but both are required for full expression. Removal of *ix* also resulted in an increase in male-characteristic compounds such as 5-T and 7-T. Once again, this result is similar to that observed in dsx^- females. These results suggest that Dsx^F and Ix function together to promote the production of female-specific pheromones.

This conclusion is further supported by the pheromone profile of dsx^F transgene males mutant for *ix*. Where one copy of the dsx^F transgene in males can induce production of the female-specific dienes 7,11-HCD and 7,11-NCD, neither of these compounds can be detected in dsx^F transgene males mutant for *ix* (Table 4, Figure 6B).

A similar dependence on *ix* was seen in production of the male-characteristic compounds 5-T and 7-T (Table 4, Figure 6B). Where the dsx^F transgene prevented production of 5-T in XY flies, $P[dsx^F26B]; ix^1/ix^{Df};+/+$ males produced nearly wild-type levels of this hydrocarbon (43.3 ng/fly). The level of 7-T was also partially restored. These results suggest that Dsx^F and Ix function together to prevent the production of male-characteristic pheromones.

DISCUSSION

Dsx^F activates *yp* expression: The best-characterized targets for *dsx* regulation are the yolk protein genes *yp-1* and *yp-2*. These genes are regulated sex specifically in the adult fat body by Dsx^F and Dsx^M. Functional dissection of a minimal fat body enhancer by An and Wensink (1995a,b) suggests that sex and tissue specificity are generated by combinatorial regulatory interactions. The minimal *yp* enhancer is composed of two elements, *o* and *r*. *o* has overlapping binding sites for three transcription factors, aef1, Dsx, and a putative bZip transcription factor. In female fat bodies, it is thought that Dsx^F binds to its target site in the minimal *o* element, while the putative bZip protein occupies a partially overlapping site. Together these two factors, plus an activator protein bound to the nearby *r* element site, turn on yolk protein expression in the fat body. In males, Dsx^M binds to the same Dsx target site in the *o* element; however, because Dsx^M has a different C-terminal domain than the female protein, it represses transcription. Repression is thought to be mediated by the larger male C-terminal domain that either prevents the bZip protein from binding to its overlapping target sequence or inactivates bound bZip protein. The low levels of yolk protein expression observed in XX and XY *dsx* mutants could be explained by the ability of the bZip protein to bind and partially

activate transcription on its own; however, in the absence of Dsx^F, full activation would not be achieved.

One prediction of this model is that ectopic expression of Dsx^F protein in males should activate transcription of the *yp* genes. Consistent with this prediction, we have found that Dsx^F turns on not only the endogenous *yp* genes, but also a LacZ reporter that contains multiple copies of *o* element from the minimal *yp* enhancer. Because Dsx^F and Dsx^M bind DNA with the same specificity and avidity (Erdman *et al.* 1996; Cho and Wensink 1997), they should compete with each other for target sites in the *yp* enhancer on essentially equal footing. Hence, when both are present, the level of *yp* gene expression would be expected to be proportional to the ratio of Dsx^F to Dsx^M. As predicted, we found that the level of *yp-1* and LacZ reporter mRNA in males carrying the dsx^F transgene depends on the number of copies of the wild-type *dsx* gene (*i.e.*, the dose of Dsx^M); when we reduced the dose of the endogenous *dsx* gene from two copies to one to none, the expression of both *yp-1* and LacZ reporter *mRNA* increased.

Dsx^F dominantly affects production of cuticular hydrocarbons: Previous studies have implicated two upstream sex determination genes, *Sxl* and *tra*, in the production of male and female pheromones. Tompkins and McRobert (1989) found that males carrying $Sxl^{M1,Pa\text{-}ra}$, a partial loss-of-function derivative of the constitutive Sxl^{M1} allele, have a female-like pheromone profile. In a more recent study, Ferveur *et al.* (1997) showed that ectopic expression of Tra^F protein in the oenocytes of males feminized the pheromone profile. The ectopic expression of either Sxl or Tra protein in XY animals would be expected to induce the female-specific expression of the known downstream genes, *dsx* and *fru*, as well as perhaps other as-yet-unidentified targets of the *Sxl* → *tra*/*tra-2* splicing cascade. While the work of Jallon *et al.* (1988) suggests that pheromone synthesis depends upon *dsx* function, there is evidence that *fru* plays some role as well (Cobb and Ferveur 1996b). The experiments presented here argue that pheromone production is under the direct control of *dsx* and is most clearly illustrated by the dominant effects of dsx^F on pheromone production in males. A single copy of the dsx^F transgene in an otherwise wild-type male is sufficient to feminize the profile of pheromones produced by the oenocytes. One of the putative male antiaphrodisiacs, 5-T, almost disappears, while the level of the other putative antiaphrodisiac, 7-T, drops to levels typically observed in wild-type females. At the same time, expression of the female-specific aphrodisiacs, 7,11-HCD and 7,11-NCD, as well as two minor female-specific hydrocarbons is induced. These findings argue that Dsx^F has a positive role in inducing the production of female pheromones, and possibly a negative role in blocking the production of male pheromones. Previous studies by Jallon *et al.* (1988) indicated that *dsx* loss-of-function mutations have the opposite effect on XX animals; they switch the pheromone profile from a female pattern to a male-like pattern (see also Figure 6). Because the major male-specific pheromones of XY animals are still present in *dsx* mutants, we suggest that production of these particular hydrocarbons represents the "default state." If this is correct, it would imply that Dsx^M is not normally required to induce the synthesis of the male antiaphrodisiacs. Dsx^M must have at least some role in downregulating the synthesis of the female aphrodisiacs because small quantities of at least three of the female-specific hydrocarbons are found in dsx^- males. However, Dsx^M can neither prevent the production of female pheromones nor induce the production of the male antiaphrodisiacs when Dsx^F is present.

To show that *dsx* is the downstream target of *Sxl* and *tra* in the sex-specific regulation of the pheromones biosynthesis, we asked whether *dsx* is epistatic to *tra*. We first examined the pheromone profile of males carrying the *hsp83* tra^F transgene. Similar to results of Ferveur *et al.* (1997), we found that the *hsp83* tra^F transgene feminizes the pheromone profile of XY animals. To determine whether this feminization is due specifically to the induction of Dsx^F by ectopic expression of Tra protein, we introduced the *hsp83* tra^F transgene into dsx^{Swe}/dsx^{Df} males. *hsp83* tra^F; dsx^{Swe}/Df males have a male pheromone profile, indicating that the dominant *dsx* allele, dsx^{Swe}, is epistatic. This finding indicates that *dsx* is the downstream target for the *Sxl* → *tra*/*tra-2* splicing cascade in pheromone biosynthesis.

While changes in the pheromone profile of XY animals induced by the dsx^F transgene suggest that Dsx^F has, at least formally, both positive and negative functions, the nature of these functions is unclear. For example, Dsx^F could activate the expression of enzyme(s) required for the synthesis of the female pheromones while blocking, albeit incompletely, the expression of an enzyme(s) required for the synthesis of male pheromones. An alternative possibility is that Dsx^F activates the expression of one or several biosynthetic enzymes that act at a branch between the male and female hydrocarbon synthesis pathways. By shunting a common precursor down the female pathway, this enzyme could simultaneously upregulate the production of female pheromones, possibly at the expense of male pheromones. In this case, Dsx^F need not repress the expression of enzymes required for male pheromone production.

Dsx^F antagonizes the masculinizing activity of Dsx^M: While Dsx functions as an activator in controlling *yp* gene expression (and probably also female pheromone synthesis), it has the opposite role, that of a "repressor," in regulating the development of male-specific morphological structures such as the sex combs, the abdominal bristles on A6, and the genitalia. As was observed for the *yp* gene expression in *hsp83-dsx*^{F} transgene males, the final phenotype of these structures depends upon the ratio of Dsx^F and Dsx^M. However, the relative level

of Dsx^F protein required to effectively antagonize the masculinizing activity of Dsx^M is higher than that required to activate the female-specific *yp* genes. Thus, in dsx^+/dsx^+ males, Dsx^F has little or no inhibitory effect on the development of these male-specific morphological structures, and transgenic animals resemble wild-type males. By contrast, relatively high levels of *yp-1* mRNA are induced by Dsx^F even in wild-type males. When there is a only a single wild-type *dsx* allele, Dsx^F is able to interfere with the masculinizing activity of the Dsx^M protein, and this results in structures that have an intersexual phenotype similar to that seen in dsx^- animals. Of course, when there is no Dsx^M (as in dsx^- males), these structures are fully feminized. These findings would be most simply explained by a model in which Dsx^F directly competes with and antagonizes the positive regulatory activity of Dsx^M (in a manner that is analogous to the competition between Dsx^F and Dsx^M in the regulation of *yp* expression). For example, Dsx^M could promote sex-comb formation by binding to target sites in the appropriate enhancers and activating gene expression, while Dsx^F would use these same target sites to block sex-comb formation. An alternative and more complicated model is that Dsx^F antagonizes Dsx^M indirectly by activating the expression of a protein(s) that represses male-specific target genes. While this model cannot be excluded at this point, it is difficult to reconcile with the known DNA-binding properties of Dsx^F and Dsx^M.

While Dsx^F can interfere with the development of male-specific morphological structures like the sex combs or the genitalia in the presence of a single wild-type *dsx* allele, this is not the case for either abdominal pigmentation or production of cVA. For both of these male-specific characteristics, ectopic expression of Dsx^F has no effect in XY animals unless both wild-type *dsx* alleles are removed. One interpretation of these findings is that these male-specific characteristics, like the sex combs or the genitalia, are positively regulated by Dsx^M and negatively regulated by Dsx^F; however, the level of Dsx^F required to antagonize the induction of these male-specific characteristics by Dsx^M is somewhat higher than is required to interfere with the development of sex combs or genitalia. While this is a plausible interpretation, it is complicated by the fact that males and females null for *dsx* have male-like pigmentation and synthesize cVA. This finding would seem to imply that male-like pigmentation and cVA synthesis represent the default state and do not require Dsx^M. This discrepancy could be resolved if only a very low level of expression of the genes specifying male pigmentation and cVA synthesis is sufficient to produce these male traits, and if these genes, like the *yp* genes, are expressed at a low level in dsx^- animals.

Behavior: *Feminine sex appeal:* The most clear-cut "behavioral" phenotype of the dsx^F transgene in dsx^+/dsx^+ males is the acquisition of feminine sex appeal; transgene males elicit vigorous courtship behavior not only from other transgene males but also from wild-type males. Because dsx^F transgene males are morphologically wild type and exhibit normal male-like courtship behavior, we presume that one source of their sex appeal is their female-like pheromone profile. It is uncertain, however, whether this is the only contributing factor. The reason for this uncertainty is that both XY and XX *dsx* mutants are courted by wild-type males (McRobert and Tompkins 1985; Villella and Hall 1996), though with less vigor than either wild-type females or dsx^F transgene males. While we detect female pheromones in both XY and XX dsx^- animals (see Table 4), the quantities are much reduced compared to those in wild-type females or in dsx^F males. If these small quantities of female pheromones are not in themselves sufficient to account for the (limited) sex appeal of the *dsx* mutant animals (see, for example, Villella and Hall 1996), then one must suppose that other unidentified feminine "attractants" (chemical or behavioral) must also be expressed in *dsx* mutants. These unidentified attractants could also contribute to the sex appeal of the dsx^F transgene males.

Irrespective of these uncertainties, the feminine sex appeal of dsx^F transgene males points to an important role for *dsx* in the etiology of sexual behavior in the fly. By inducing expression of known aphrodisiacs (and perhaps other "attractants"), dsx^F appears to be responsible for the sex appeal of wild-type females. Although not a behavior in itself, feminine sex appeal plays a critical role in successful sexual behavior in that it elicits courtship by wild-type males. Obviously, in males this requires a system that specifically recognizes dsx^F-induced feminine attractants and then sets in motion male courtship behavior.

Sexual behavior: In an otherwise wild-type male, the dsx^F transgene does not seem to have any effect on the system that senses and responds to feminine sex appeal. However, when the dose of the wild-type *dsx* gene is reduced, abnormalities in male courtship behavior are observed. Although $hsp83dsx^F;dsx^+/dsx^-$ males will court females, they do so less frequently and less aggressively than control dsx^+/dsx^- males. Even more severe reductions in the courtship index were evident in $hsp83dsx^F$ pseudofemales ($hsp83dsx^F;dsx^-$). These animals court females only infrequently and exhibit, at most, only early courtship behaviors. Perhaps even more remarkable, the dsx^F pseudofemales allow themselves to be mated by wild-type males. In this female-like behavior, they differ in two respects from wild-type females. First, the time to copulation is much longer. This is due, at least in part, to avoidance behaviors such as wing flicking and moving around the mating chamber. Second, during copulation, the dsx^F pseudofemales actively attempt to dislodge the male. It may be interesting in this respect that very similar abnormalities in female behavior are observed in *dsf* mutant females (Finley *et al.* 1997). Although genetic studies initially suggested that *dsf* was

part of a separate *tra*/*tra-2* pathway independent of *fru* and *dsx*, molecular analysis of *dsf* has not revealed any sex-specific regulation (Finley *et al.* 1998). To account for the sex-specific behavioral phenotypes of *dsf*, Finley *et al.* (1998) speculate that *dsf* may be part of a sex-specific complex that itself is regulated by *tra*/*tra-2*. If this is correct, then the "*dsf* complex" should still be in the "male" mode in *hsp83-dsx*^{F} pseudofemales. This could explain why the behavior of *hsp83-dsx*^{F} pseudofemales is not completely feminized. Alternatively, *fru* expression is still in the "male" mode and might interfere with feminization.

The fact that expression of Dsx^F in XY animals reduces or eliminates male courtship behavior and results in a concomitant acquisition of some female-like behaviors indicates that the *dsx* gene must play a role in sexual behavior beyond simply producing the feminine sex appeal. Our findings would be consistent with the idea that in XX animals, Dsx^F normally functions together with *dsf* to generate behavioral patterns appropriate for females. Because *dsx* does not seem to be required in XY animals to generate most male courtship behaviors (Villella and Hall 1996), *hsp83dsx*^{F} may suppress male behavior in transgenic XY animals by some indirect mechanism. For example, Dsx^F could prevent the formation or proper function of cells critical for sensing or responding to stimuli from females. Alternatively, it might interfere with male courtship behavior because it activates female behavioral circuits.

What is the role of *intersex*? Previous studies have shown that *ix* is required for normal female development, but is dispensable in males (Baker and Ridge 1980; Chase and Baker 1995). From these earlier studies alone, it is unclear whether *ix* is regulated directly by the *Sxl* → *tra* splicing cascade or by *dsx*, or constitutively expressed in both sexes. Because *ix* is required for the induction of both *yp* mRNA synthesis and female pheromones in *dsx*^{F} transgene males, it would appear that *ix* expression is not directly dependent upon the *Sxl* → *tra* splicing cascade. While we cannot exclude the possibility that Dsx^F induces *ix* expression in XY animals, our results would be most easily explained by nonsex-specific constitutive expression of *ix*.

How does *ix* function in female sexual differentiation? With respect to *yp* expression and probably also pheromone production, our results argue that *ix* is an essential cofactor for Dsx^F, enabling Dsx^F to function as a positive regulator. Two different mechanisms could account for the effects of *ix* mutations on *yp* expression in normal females and in *dsx*^{F} transgene males. *ix* could correspond to the unknown bZip transcription factor that is postulated to bind adjacent to Dsx in the minimal *o* element enhancer. An alternative and seemingly more likely mechanism is that Ix physically interacts with and/or modifies Dsx^F to potentiate its positive regulatory activities. A potentiating function mediated, perhaps, through interactions with the female-specific domain of the Dsx protein, would also account for the role of *ix* in facilitating the repression of male-specific developmental pathways by Dsx^F. These questions will ultimately be resolved by the cloning and characterization of the *ix* gene.

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