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hermaphrodite and *doublesex* function both dependently and independently to control various aspects of sexual differentiation in *Drosophila*

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SUMMARY

The *hermaphrodite (her)* gene is necessary for sexual differentiation in *Drosophila*. Our characterization of *her*'s zygotic function suggests that one set of female-specific terminal differentiation genes, the *yolk protein (yp)* genes, is transcriptionally activated by two separate pathways. One is a female-specific pathway, which is positively regulated by the female-specific *doublesex* protein (DSX^F). The other is a non-sex-specific pathway, that is positively regulated by HER. The HER pathway is prevented from functioning in males by the action of the male-specific *doublesex* protein (DSX^M). The HER and DSX pathways also function independently to control downstream target genes in the precursor cells that give rise to the vaginal teeth and dorsal anal plate of females, and the lateral anal plates of males. However, a female-specific pathway that is dependent on both DSX^F and HER controls the female-specific differentiation of the foreleg bristles and tergites 5 and 6, and the male-specific differentiation of these tissues does not require the suppression of HER's function by DSX^M.

Key words: *hermaphrodite* (*her*), *doublesex* (*dsx*), *yolk protein* (*yp*), Sexual differentiation, *Drosophila melanogaster*

INTRODUCTION

A hierarchy of regulatory genes controls somatic sex determination and differentiation in *Drosophila melanogaster* (reviewed, for example, by Burtis, 1993; Burtis and Wolfner, 1992; Cline and Meyer, 1996; McKeown, 1992; Parkhurst and Meneely, 1994). There are two branches in the hierarchy downstream of the *transformer (tra)* gene. One branch contains the *doublesex (dsx)* gene (Baker and Ridge, 1980) and the other the *fruitless (fru)* gene (Ryner et al., 1996). *dsx* is required for all known aspects of somatic sexual differentiation outside of the CNS (reviewed by Burtis and Wolfner, 1992) as well as some aspects of sexual differentiation in the CNS (Taylor and Truman, 1992; Villella and Hall, 1996). The sex determination function of *fru* appears to be required for the sexual differentiation of only a small set of cells in the CNS concerned with male sexual behaviors (Gailey et al., 1991; Hall, 1994; Ito et al., 1996; Lawrence and Johnston, 1986; Ryner et al., 1996; Taylor et al., 1994).

At the bottom of the *dsx* branch of the somatic sex determination hierarchy are the *dsx, hermaphrodite (her)* (Pultz and Baker, 1995) and *intersex (ix)* (Chase and Baker, 1995) genes. *dsx* encodes sex-specific transcription factors (Burtis et al., 1991). The female-specific DSX protein (DSX^F) acts together with the *her* (Pultz and Baker, 1995) and *ix* (Erdman et al., 1996) gene products to inhibit male differentiation and activate female differentiation in females and, conversely, the male-specific DSX protein (DSX^M) acts to inhibit female differentiation and activate male differentiation in males (Jursnich and Burtis, 1993; Taylor and Truman, 1992; Villella and Hall, 1996; reviewed by Burtis, 1993; McKeown and Madigan, 1992). The best-characterized terminal differentiation genes that are sex-specifically regulated by the somatic sex determination hierarchy are the *yolk protein (yp)* genes *yp1, yp2* and *yp3*. The transcription of *yp1* and *yp2* in fat body cells is directly activated by DSX^F in females and inhibited by DSX^M in males, through the binding of the DSX proteins to the fat-body-specific enhancer (FBE) of *yp1* and *yp2* (reviewed by Bownes, 1994).

Previous genetic results indicated that the zygotic function of *her* acts together with, or downstream of, *dsx*, since *her* and *dsx* mutations have equivalent phenotypes in females, yet neither the transcription nor the splicing of the *dsx* pre-mRNAs is affected in *her* mutants (Pultz and Baker, 1995). Recently, we molecularly characterized the *her* gene and showed that (1) *her* encodes a single protein with C_2H_2-type zinc fingers, (2) *her* is transcribed throughout all developmental stages in both sexes, and (3) the splicing of the *her* pre-mRNA is not sex-specific, suggesting that *her* is not sex-specifically regulated at the levels of transcription or splicing (Li and Baker, 1998). These results suggest that *her* is expressed non-sex-specifically and is not a downstream target of *dsx*. The available data thus support a conclusion that *her* acts together with *dsx* in sexual differentiation in females.

It is unclear whether the zygotic function of *her* is also involved in sexual differentiation of males. It was reported that there are extra bristles on the sixth sternite of *her* mutant males and that males that die as pharate adults often have rotated

genitalia (Pultz et al., 1994). These mutant male phenotypes resemble the phenotypes of males that have partially lost the function of *dsx* (Pultz et al., 1994). Thus it was suggested that *her* has a sexual differentiation function in males, presumably acting together with DSX^M (Pultz and Baker, 1995; Pultz et al., 1994). However, the phenotype of *her* mutant males could also be interpreted as segmental transformation (Pultz et al., 1994) since *her*, in addition to its specific roles in sex determination, also has a sex-independent essential function (Pultz et al., 1994).

We report here a characterization of the zygotic sex determination function of the *her* gene. We show that HER non-sex-specifically activates the transcription of the *yp* genes in fat body cells, independent of DSX^F, and HER's function in activating *yp* transcription is inhibited in males by DSX^M. We provide genetic evidence that HER and DSX^F also function independently to bring about female-specific differentiation in several other sexual dimorphic tissues. In addition, we show that, in some tissues, HER and DSX^F function dependently to control female-specific differentiation. Finally, we show that, in one tissue in males, HER and DSX^M are both required for normal sexual differentiation.

MATERIALS AND METHODS

Fly stocks

Flies were raised on standard corn meal food. Experiments were done at the temperature indicated. All mutations not referenced in the text and the nomenclatures of standard *Drosophila* genetics can be found in Lindsley and Zimm (1992). The *her* alleles used were previously described (Pultz et al., 1994).

Northern analysis

Northern analyses of *yp2* and *yp3* were performed using 20-30 μg total RNA per lane. *yp2* and *yp3* [^{32}P]DNA probes were made by random primer labeling. RNAs were electrophoresed, transferred and probed using standard procedures. The *rp49* [^{32}P]DNA probe was made using an asymmetrical PCR method (Innis et al., 1990). Exposure of the blots and quantitation of the signals were done using the BioRad PhosphorImager system.

CPRG assay

The *lacZ* activities were measured according to the previously published protocol (Coschigano and Wensink, 1993) with the following modification. Depending on the β-galactosidase level of each genotype, 15-20 females were homogenized in 1 or 0.5 ml and 15-20 males in 0.5 ml lysis buffer, and 1-200 μl lysate was used to measure the *lacZ* activity.

Statistical analysis

For simple *t*-test, we used a pocket calculator. For analysis of variance (ANOVA) of the data in Table 2, we used the computer program StatView (Abacus Concepts, Inc.). To examine if there is interaction between *dsx* and *her* in females, we did ANOVA using logarithmically transformed primary data, since if there is no interaction between *dsx* and *her*, the expected value in *her/+; dsx/+* females is the product of the values in *her/her; dsx/+* females and *her/+; dsx/dsx* females.

RESULTS

her activates the *yps* in females, but does not repress them in males

Previous results indicated that *dsx* and *her* may function together to control somatic sexual differentiation. Since the only characterized target genes of *dsx* are the *yp* genes (reviewed by Bownes, 1994), we investigated whether *her* also regulates the expression of the *yp* genes and whether *her* functions similarly to *dsx* in their regulation.

We initially used northern analysis to examine the effects of *her* on expression of the *yps*. Since the complete loss of *her* function is lethal, the temperature-sensitive allele her^1 was used. At 25°C, her^1 flies are intersexual and have severely reduced viability while, at 18°C, they are morphologically normal, and have wild-type viability and fertility (Pultz et al., 1994). As shown in Fig. 1A, there is a 10-fold activation of *yp2* expression by her^+, since *her* females raised at 25°C showed a 10-fold reduction of *yp2* transcript levels as compared to wild-type females and their her^1/+ sisters. This is comparable

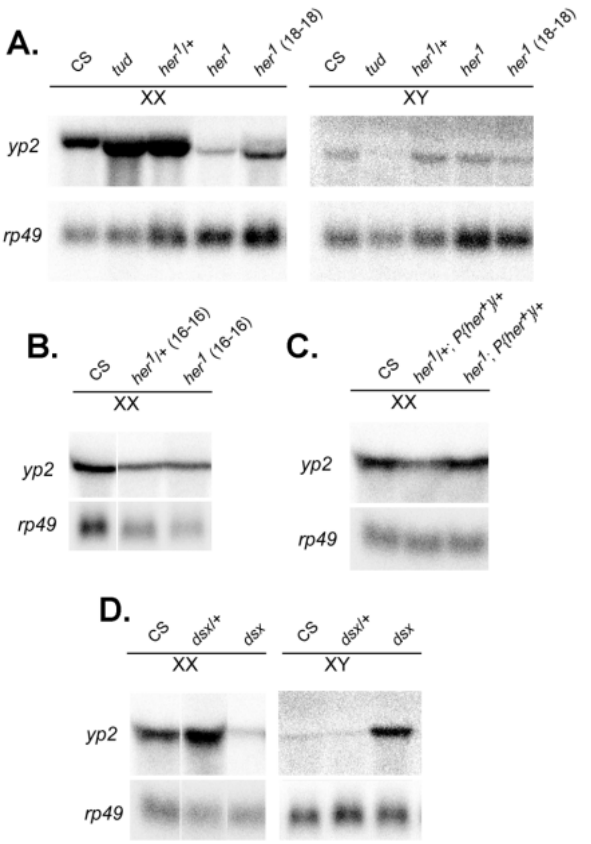

Fig. 1. The *her* and *dsx* genes control *yps* expression. (A-D) Northern analysis of *yp2* expression. Total RNAs of adult flies were used (20-30 μg/lane) for the analysis (see Materials and Methods). *yp2* indicates the *yp2* RNA level and *rp49* indicates the *rp49* RNA level that serves as the control for the amount of RNA loaded in each lane. In A and D, the signals showing the XY *yp2* RNA levels were obtained by longer exposure of the XY blots than the XX blots. Except as indicated, the flies were raised at 25°C and aged as adult at 25°C for 3-4 days. The first number in the parenthesis indicates the temperature (°C) at which the flies were raised and the second number indicates the temperature at which the adult flies were aged. The complete genotypes are as follows. CS, Canton-S wild-type flies; *tud*: maternal genotype is *tud*. her^1/+: *b* her^1/*CyO*. her^1: *b* her^1/*b* her^1. her^1/+; *P{*her^+*}*/+: *b* her^1/+; *P{*her^+*}*/+. *dsx*/+: *dsx* p^p/+. *dsx*: *dsx* p^p/*dsx* p^p. *P{*her^+*}* is a transgene containing the her^+ genomic DNA fragment B17 (Li and Baker, 1998).

We initially used northern analysis to examine the effects of *her* on expression of the *yps*. Since the complete loss of *her* function is lethal, the temperature-sensitive allele her^1 was used. At 25°C, her^1 flies are intersexual and have severely reduced viability while, at 18°C, they are morphologically normal, and have wild-type viability and fertility (Pultz et al., 1994). As shown in Fig. 1A, there is a 10-fold activation of *yp2* expression by her^+, since *her* females raised at 25°C showed a 10-fold reduction of *yp2* transcript levels as compared to wild-type females and their her^1/+ sisters. This is comparable

to the activation effect of the dsx^+ gene in females (Fig. 1D). The 10-fold activation by her^+ could be an underestimate, since her^1 may not be null at 25°C with respect to its function in *yp* expression. The level of *yp2* transcript is restored to the wild-type level in *her* mutant females raised at 25°C when they also carry a transgene *(P{her⁺})* containing a wild-type copy of the *her* gene (Fig. 1C), demonstrating that it is the her^1 mutation, rather than some other mutation on the her^1 chromosome, that causes the reduction in *yp2* transcript level. To our surprise, *yp2* expression was also reduced 10-fold in the her^1 homozygous females raised at 18°C (Fig. 1A). However, when grown at 16°C, her^1 females have levels of *yp2* expression comparable to that seen in wild-type females (Fig. 1B). These results indicate that *yp2* expression is more sensitive to the level of *her* function than is external sexual morphology.

In *her* males, the *yp2* transcript level remains unchanged (Fig. 1A). This is in striking contrast to *dsx* males where the *yp2* level is increased 20-fold compared to that of wild-type males and *dsx*/+ brothers, consistent with previous findings that DSX^M functions to repress the transcription of the *yps* (Fig. 1D) (Coschigano and Wensink, 1993; Ota et al., 1981). This result reflects a fundamental difference between the *her* and *dsx* functions in males, rather than a leakiness of her^1 (see below).

Since in wild-type females the *yps* are expressed both in fat body cells and in ovarian follicle cells (Brennan et al., 1982; Postlethwait et al., 1980), and the ovaries are often underdeveloped in *her* mutant females, the reduced level of *yp2* transcripts in *her* mutant females could be due to the small size of the ovaries in these females, rather than reduced *yp2* expression in the fat body cells. However, this is not the case. The daughters of females homozygous for the *tudor* mutation have no germlines and therefore have only rudimentary ovaries (Boswell and Mahowald, 1985). In the daughters of *tud* mothers, the level of *yp2* transcript is comparable to that in wild-type females (Fig. 1A), demonstrating that the majority of the *yp2* transcripts detected by northern blots are synthesized in female fat body cells.

The loss of *yp2* transcripts in her^1 females could be due to either an indirect effect, if *her* has some role in the development of fat body tissues per se, or a direct role of *her* in regulating the *yps* expression. We believe *her*'s effect on the *yps* expression is likely to be direct, since (1) the her^1 mutation has only a 2-fold effect on the expression of a reporter gene pML-58 (Fig. 2) that is specifically expressed in female fat body cells (see below); (2) the her^1 mutation has no effect on the expression of a reporter gene FBE-*hsp70 promoter-lacZ* (Garabedian et al., 1986) that is also specifically expressed in female fat body cells (data not shown); and (3) shifting of the *her* ts mutant adult females to a non-permissive temperature causes a reduction of the expression of a *yp*-reporter gene pCR1 (Fig. 2) (see below).

The expression of the *yp1* and *yp3* genes is regulated the same way by *dsx* and *her* as is the *yp2* gene. This was shown by probing the same set of the northern blots (Fig. 1A-D) with probes made from *yp1* and *y3* genomic DNAs (data not shown).

Based on these results, we conclude that HER is required, like DSX^F, for the activation of the *yps* in female fat body cells. But, in contrast to DSX^M, HER is not required for the inhibition of the *yps* expression in males.

her regulates the transcription of the *yp* genes

The reduction of *yp2* transcripts in *her* mutant females could be due to the involvement of *her* in the regulation of *yp2* transcription or *yp2* RNA stability. To distinguish between the two possibilities, we employed the *yp* reporter gene pCR1 (Fig. 2). In the pCR1 construct, the intergenic regulatory region of the divergently transcribed *yp1* and *yp2* genes remains intact while the coding sequences of *yp1* and *yp2* are replaced by the *Drosophila melanogaster Adh* and the *Escherichia coli lacZ* genes, respectively (Fig. 2; Lossky and Wensink, 1995). The 3′UTR of the *Drosophila melanogaster α-1* tubulin gene is fused to the 3′ end of the *lacZ* gene (Logan and Wensink, 1990). We used β-galactosidase activity to indicate the level of pCR1 transcripts**.** The effects of *her* and *dsx* on the expression of the *lacZ* gene of pCR1 were in all cases comparable to their effects on the *yps* expression as monitored by northern blots, demonstrating that *her,* like *dsx,* controls the *yps* expression at the level of transcription, rather than RNA stability.

Thus DSX^F increases pCR1 activity 10-fold in females (Table 1, compare rows 1 and 2, $P<0.02$, by *t*-test hereafter except as noted; see Materials and Methods for details of the enzymatic assay), whereas DSX^M represses pCR1 activity about 70-fold in males (Table 1, compare rows 3 and 4, $P<0.01$). When DSX^M is present, the transcription of pCR1 is most likely completely turned off, since the level of β-

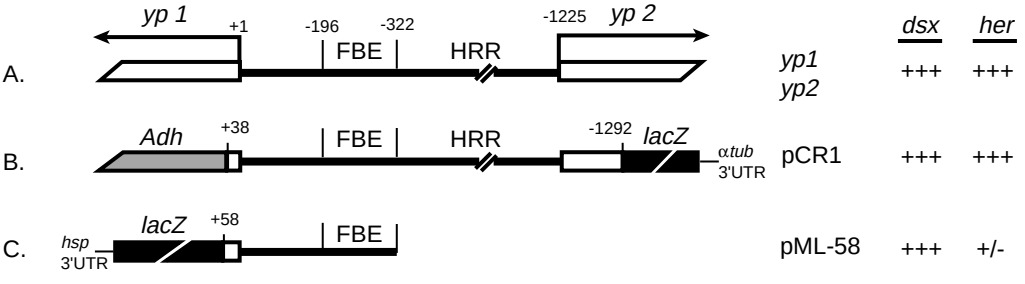

Fig. 2. *yp*-reporter genes and their responses to *dsx* and *her* regulation. (A) Simplified view of *yp1* and *yp2* The numbers indicate the nucleotide positions of the intergenic region of *yp1* and *yp2*. Position +1 and −1225 are the transcription start sites of *yp1* and the *yp2*, respectively. The FBE (from position −196 to −322) is the fat body enhancer element (Garabedian et al., 1986). HRR (from position −322 to −1225) is the *her*-responsive region. Arrow indicates the direction of transcription. (B) The pCR1 reporter gene structure has been previously reported (Lossky and Wensink, 1995). *Adh* is the alcohol dehydrogenase gene of *Drosophila melanogaster*. *lacZ* is the β-galactosidase gene of *Escherichia coli*. The αtub 3′UTR indicates the 3′ untranslated region of α-1 tubulin gene of *Drosophila melanogaster*. (C) The pML-58 reporter gene construct is provided by M. Lossky and P. Wensink. *hsp* 3′UTR indicates the 3′UTR of the *hsp70* gene of *Drosophila melanogaster*. The degree of response to the regulation by *dsx* and *her* is indicated, which is based on the data in Fig. 1 for A, the data in Table 1 for B and the data in Table 3 for C.

Table 1. *dsx* and *her* regulate pCR1 expression

Row*		Genotypes†	*lacZ* activity (mean)‡	s.d.§	*n*¶
1	pCR1;	*dsx*/+	7.93×10^{-1}	1.31×10^{-1}	2
2	pCR1;	*dsx*	7.80×10^{-2}	8.23×10^{-3}	2
3	pCR1/Y;	*dsx*/+	4.38×10^{-4}	2.08×10^{-5}	2
4	pCR1/Y;	*dsx*	2.92×10^{-2}	2.92×10^{-3}	2
5	pCR1/+;	*her*/+; *P{her$^+$}*	6.50×10^{-1}	1.44×10^{-2}	2
6	pCR1/+;	*her*; *P{her$^+$}*	7.19×10^{-1}	5.50×10^{-2}	2
7	pCR1;	*her*/+	1.73×10^{0}	4.44×10^{-2}	2
8	pCR1;	*her*	1.64×10^{-1}	1.69×10^{-3}	2
9	pCR1/Y;	*her*/+	2.91×10^{-4}	4.64×10^{-5}	2
10	pCR1/Y;	*her*	3.69×10^{-4}	1.36×10^{-5}	2
11	CS F		3.19×10^{-4}	5.79×10^{-7}	2
12	CS M		1.54×10^{-4}	1.41×10^{-5}	2
13	pCR1/+;	*her*/+	6.50×10^{-1}	3.31×10^{-2}	2
14	pCR1/+;	*her*	2.36×10^{-1}	2.08×10^{-2}	2
15	pCR1/+;	*her*/+	2.68×10^{-1}	3.10×10^{-2}	2
16	pCR1/+;	*her*	3.20×10^{-1}	2.21×10^{-2}	2

*Rows 1-4, 5-6, 7-10, 11-12, 13-14 and 15-16 form independent groups, respectively. Each group contains results from sibs, thus formally only within a group data can be compared. The flies in rows 13-16 are sibs, but those in rows 13-14 and 15-16 were treated at different temperatures (see below). All flies were raised at 25°C and aged as adults at 25°C except that (1) the flies in rows 7-10 were raised at 18°C and aged as adults at 25°C, (2) the flies in rows 13-14 were raised at 18°C and aged as adults at 18°C, then shifted to 25°C for 1 day, (3) the flies in rows 13-14 were raised at 18°C and aged as adults at 18°C.

†The complete genotypes in rows 1-16 are as follows. The pCR1 reporter gene is X-linked. 1, pCR1/pCR1; *dsx p^p/MKRS, ry*. 2, pCR1/pCR1; *dsx p^p/dsx p^p*. 3, pCR1/*B^s*Y; *dsx p^p/MKRS, ry*. 4, pCR1/ *B^s*Y; *dsx p^p/dsx p^p*. 5, pCR1/+; *b her^1/(CyO/SM1); P{her$^+$}/ry*. 6, pCR1/+; *b her^1*; *P{her$^+$}/ry*. 7, pCR1/pCR1; *b her^1/SM1; ry*. 8, pCR1/pCR1; *b her^1*; *ry*. 9, pCR1/Y; *b her^1/SM1; ry*. 10, pCR1/Y; *b her^1*; *ry*. 11, Canton-S wild-type female. 12, Canton-S wild-type male. 13 and 15, pCR1/+; *b her^1/Gla; ry*/+. 14 and 16, pCR1/+; *b her^1/SM1, her^2; ry*/+.

‡The unit for *lacZ* activity is ΔOD_{574}/min/mg fly, based on the CPRG assay (see Materials and methods).

§Standard Deviation.

¶The number of replicas.

galactosidase activity in *dsx*/+ males is similar to that seen in wild-type (Canton-S) females that lack pCR1 and only 3-fold higher than the level seen in wild-type males that lack pCR1 (Table 1, compare rows 3, 11 and 12). The observation that *dsx* has the same effect on the pCR1 activity as it has on the levels of the *yps* transcripts indicates that *dsx* regulates the *yps* expression at the level of transcription, rather than the level of RNA stability, consistent with previous findings (Coschigano and Wensink, 1993).

Similarly, *her*$^+$ activity is also required in females for the transcriptional activation of the *yps*, rather than the stability of their transcripts. Thus *her*$^+$ activates pCR1 10-fold (Table 1, compare rows 7 and 8, $P<0.001$), comparable to what was seen by northern analysis. Consistent with the northern result, the reduction of pCR1 activity in *her^1* mutant females is not due to the underdevelopment of ovaries in *her^1* females, since the *lacZ* activity from ovaries amounts to only 1% of the total *lacZ* activity in pCR1; *her*$^+$; *dsx*$^+$ females (data not shown). That these effects are due to the *her^1* mutation is shown by the fact that one copy of the wild-type *her* gene as a transgene *(P{her$^+$})* restores the *lacZ* activity to the wild-type level in *her^1* females (Table 1, compare rows 5 and 6, $P>0.2$). *her^2* is another ts mutant allele of *her*, weaker than *her^1* (Pultz et al., 1994). At permissive temperature (18°C), the pCR1 activity remains the same in *her^1/her^2* mutant females and their wild-type sisters (Table 1, compare rows 15 and 16, $P>0.1$). However, when the *her^1/her^2* mutant females were shifted to non-permissive temperature (25°C) for a day, the pCR1 activity is reduced 3-fold compared to their wild-type sisters (Table 1, compare rows 13 and 14, $P<0.01$). This result suggests that the effect of *her^1* mutation on the pCR1 activity is not the special property of the *her^1* allele, but rather a property of *her* mutations per se.

In contrast to *dsx*, *her* is not required for the repression of the *yps* in males. Thus, consistent with the northern result, the pCR1 activity remained the same in *her^1* homozygous males and their *her^1*/+ male sibs (Table 1, compare rows 9 and 10, $P>0.1$).

In conclusion, our results demonstrate that *her*, like *dsx*, activates the transcription of the *yps* in females through the intergenic region of *yp1* and *yp2*.

The DSX proteins are the major female-specific and male-specific regulators of the *yps*

The perceptions that derive from the above experiments concerning the roles of *dsx* and *her* in regulating the transcription of the *yp* genes suggest that both genes function in the activation of the *yp* genes in females, but that only *dsx* functions in males, where it acts to repress the *yps* expression. However, consideration of the quantitative aspects of the data from these experiments indicates that this interpretation is incorrect. In particular, the data with respect to the roles of DSX^M in males and DSX^F in females indicate that *dsx* function can account for all of the difference between the sexes in the levels of the *yps* expression. These findings with regard to *dsx* clearly contradict the idea that there is a female-specific role for *her* in the activation of the *yp* genes. Below we present the data that lead to this contradiction and suggest two alternative views of the role of *her* in regulating the *yps* expression that are consistent with these results.

The argument that *dsx* is the major, if not the only, sex-specific regulator of the *yp* genes derives from the analysis of the transcriptional regulation of pCR1. The pCR1 *lacZ* activity in *dsx* /+ females is about 2000-fold higher than in *dsx* /+ males (no expression of the *yp* genes) (Table 1, compare rows 1 and 3, $P<0.001$). However, the difference is only about 2.6-fold between the *dsx* homozygous female and male sibs (Table 1, compare rows 2 and 4, $P<0.02$). Since females homozygous for the X-linked pCR1 transgene were assayed, the 2.6-fold difference in the pCR1 activity between the *dsx* females and males is largely, if not entirely, due to the 2-fold difference in the gene dosage of pCR1 between females (two copies of the pCR1 transgene) and males (one copy of the pCR1 transgene). This conclusion was confirmed by our data that the *lacZ* activity is the same in *dsx* mutant females and males that carry only one copy of the pCR1 transgene (data not shown). These results are consistent with previous data that the activity of the *lacZ* gene under the control of the *hsp70* promoter and the fat body enhancer (FBE) of *yp1* and *yp2* is the same in *dsx/dsx* females and males (Coschigano and Wensink, 1993). Thus, these results demonstrate that, in the absence of *dsx*, the *yp* genes are expressed at the same levels in both sexes.

In considering these results, it is important to note that two factors contribute to making the levels of the *yps* expression

equivalent in *dsx* mutant males and females. First, the expression level of the *yp* genes is elevated in *dsx* males (compared to wild-type males), due to the absence of repression by DSX^M. Second, the expression level of the *yp* genes in *dsx* females is reduced, due to the absence of activation by DSX^F. Thus in both *dsx* mutant males and females, there are significant levels of expression of the *yp* genes, and these levels are equivalent in the two sexes.

There are two ways that we can see to reconcile these observations with regard to *dsx* with the observation that *her* appears to control the expression of the *yp* genes female-specifically. One model is that *her* does function female-specifically, but that its female-specific function is dependent on DSX^F. The second model is that *her* functions sex-independently to activate the expression of the *yp* genes, but that its action in males is precluded by DSX^M's repression of the *yp*'s expression. These two models make different predictions as to the effects expected of *her* mutants in *dsx* mutant backgrounds. If the first model is correct, the presence or absence of *her* should have no effect on the *yps* when DSX^F is absent. If the second model is correct, *her* should be able to activate the *yps* in *dsx* mutant males where DSX^M is absent. Experiments that distinguish the two models are described below.

her is a non-sex-specific activator of the *yps*

To examine the effects of *her* on the *yps* expression in the absence of *dsx* function, we used the pCR1 reporter gene. To obtain sufficient mutant flies in a relatively short time period, we increased the viability of *her/her; dsx/dsx* flies at 25°C by using a chromosome that contains the her^1 allele and a copy of the *hsp70* promoter-*her* cDNA transgene named $hsp\text{-}her^{\#11}$ that rescues the lethality and external phenotypes of *her* mutants, but not their defect in the *yps* expression (Table 2, compare rows 1 and 3, $P<0.0001$; data not shown).

To test whether HER activates the *yps* expression independent of DSX^F in females, we examined the responsiveness of pCR1 to HER regulation in the absence of DSX^F. When DSX^F is absent and HER is present in females (*her/+; dsx/dsx* females), the pCR1 activity is increased 7-fold as compared to the *her/her; dsx/dsx* female sibs (Table 2, compare rows 2 and 4, $P<0.01$). Since HER, even when DSX^F is present, can only activate pCR1 14-fold (Table 2, compare rows 1 and 3, $P<0.0001$), our data suggest that the activation function of HER is not strongly dependent on DSX^F.

To test whether *her* activates the *yps* expression in males in the absence of the inhibition by DSX^M, we examined the responsiveness of pCR1 to *her* regulation in the absence of DSX^M. In males, when DSX^M is present, pCR1 is not expressed whether HER is present (*her/+; dsx/+* males) or absent (*her/her; dsx/+* males) (Table 2, compare rows 5 and 7, $P>0.9$). However, in males without DSX^M, pCR1 is expressed and the pCR1 activity is 5-fold higher when HER is present (*her/+; dsx/dsx* males) than when HER is absent (*her/her; dsx/dsx* males) (Table 2, compare rows 6 and 8, $P<0.0001$). This finding suggests that wild-type *her* function is normally present in males and capable of activating the transcription of the *yp* genes, but its activity is normally overridden by the inhibitory function of DSX^M.

In conclusion, we have discovered that there are two separate pathways for the activation of the *yps*. One is the female-specific activation of the *yps*, which is DSX^F-dependent. The other is the non-sex-specific activation of the *yps*, which is HER-dependent, DSX^F-independent and inhibited by DSX^M. Our results also suggest that *her* has the same biological function in both sexes, providing further evidence that the expression of *her* is independent of the sex determination hierarchy.

Table 2. *dsx* and *her* act independently in females, and *dsx* inhibits *her* in males

Row*	Genotypes†		*lacZ* activity (mean)‡	s.d.§	*n*¶
1	pCR1; *her/+*	*dsx/+*	8.86×10^{-1}	4.06×10^{-2}	4
2	pCR1; *her/+*	*dsx*	3.89×10^{-2}	2.06×10^{-2}	10
3	pCR1; *her*	*dsx/+*	6.32×10^{-2}	1.32×10^{-2}	8
4	pCR1; *her*	*dsx*	5.98×10^{-3}	2.18×10^{-3}	4
5	pCR1/Y; *her/+*	*dsx/+*	8.20×10^{-4}	3.58×10^{-4}	4
6	pCR1/Y; *her/+*	*dsx*	1.93×10^{-2}	5.92×10^{-3}	8
7	pCR1/Y; *her*	*dsx/+*	8.07×10^{-4}	8.98×10^{-5}	4
8	pCR1/Y; *her*	*dsx*	4.12×10^{-3}	1.23×10^{-3}	8

*Rows 1-8 contain results from sibs. All flies were raised at 25°C and aged as adults at 25°C.

†The complete genotypes in rows 1-8 are as follows. 1, pCR1/pCR1; her^1 or her^1 $hsp\text{-}her^{\#11}$ */CyO*; $dsx\ p^p/MKRS$, *ry*. 2, pCR1/pCR1; her^1 or her^1 $hsp\text{-}her^{\#11}$ */CyO*; $dsx\ p^p/dsx\ p^p$. 3, pCR1/pCR1; her^1/her^1 $hsp\text{-}her^{\#11}$; $dsx\ p^p/MKRS$, *ry*. 4, pCR1/pCR1; her^1/her^1 $hsp\text{-}her^{\#11}$; $dsx\ p^p/dsx\ p^p$. 5, pCR1/Y; her^1 or her^1 $hsp\text{-}her^{\#11}$ */CyO*; $dsx\ p^p/MKRS$, *ry*. 6, pCR1/Y; her^1 or her^1 $hsp\text{-}her^{\#11}$ */CyO*; $dsx\ p^p/dsx\ p^p$. 7, pCR1/Y; her^1/her^1 $hsp\text{-}her^{\#11}$; $dsx\ p^p/MKRS$, *ry*. 8, pCR1/Y; her^1/her^1 $hsp\text{-}her^{\#11}$; $dsx\ p^p/dsx\ p^p$.

‡-¶Same as in Table 2.

DSX^F and HER can activate the *yps* independently in females

We have shown that, in females, HER can act independently of DSX^F. We have also asked whether the DSX^F-directed female-specific activation of the *yps* is dependent on the HER-directed non-sex-specific activation pathway, and whether there is an interaction between DSX^F and HER in females.

Our data showed that DSX^F is still able to activate the *yps* in *her/her* females. DSX^F increases the pCR1 activity by 11-fold in *her/her* females (Table 2, compare rows 3 and 4, $P<0.0001$). DSX^F, even when HER is present, can only activate the *yps* 20-fold (Table 2, compare rows 1 and 2, $P<0.0001$). Thus, these results suggest that the activation function of DSX^F is not strongly dependent on the activation function of HER.

Our data also show that there is a weak interaction between DSX^F and HER in females. We used the *lacZ* activity of pCR1 in the XX; her^1/her^1; *dsx/dsx* females as a baseline for comparisons, since this reflects the pCR1 activity in the absence of both *dsx* and *her*. When dsx^+ is added (XX; *her/her; dsx/+*), the *lacZ* activity of pCR1 is increased 11-fold (Table 2, compare rows 3 and 4, $P<0.0001$). If her^+ alone is added (XX; *her/+*; *dsx/dsx*), the pCR1 activity is increased 7-fold (Table 2, compare rows 2 and 4, $P<0.01$). When both her^+ and dsx^+ are added (XX; *her/+*; *dsx/+*), the pCR1 activity is increased 150-fold (Table 2, compare rows 1 and 4, $P<0.0001$). If *her* and *dsx* act independently, one would expect a 77-fold increase, rather than a 150-fold increase, in the pCR1 activity when both her^+ and dsx^+ are added. To examine whether the differences between the observed and the expected values are statistically significant, we did analysis of variance (ANOVA). Our results suggest that there is an interaction between *her* and

dsx in the activation of the *yps* in females (p <0.02; see Materials and Methods for details). However, this interaction is weak and is not obligatory for the activation functions of either *dsx* or *her*.

The DNA sequences mediating *her* regulation

Our conclusion that *her*'s activation of the *yps* appears to be largely independent of *dsx* is qualified by the fact that we were not able to use null alleles of *her* in these experiments. To further address the independence of *her* and *dsx*, we asked whether the sequences that are necessary and sufficient to mediate *dsx*'s regulation of the *yps* expression are also sufficient to mediate *her*'s regulation of these genes. Previous experiments showed that, in vitro, the DSX^F and DSX^M proteins bind to the 127 bp FBE element, but not to other sequences of the intergenic region of *yp1* and *yp2* (Burtis et al., 1991; Coschigano and Wensink, 1993). In vivo, the FBE element is sufficient to direct the DSX-dependent, sex-specific expression of a reporter gene from a heterologous promoter (Coschigano and Wensink, 1993).

To address this topic, we used the *yp*-reporter gene pML-58 (Fig. 2). In pML-58, the DNA sequences from nucleotide position −322 to +58 of the *yp1* gene are fused to the *E. coli lacZ* gene whose 3′ end is fused to the 3′UTR of the *hsp70* gene (position +1 and −1225 are the transcription start sites of *yp1* and *yp2*, respectively) (Fig. 2). Since the DNA sequence from nucleotide position −196 to −322 is the FBE element (Garabedian et al., 1986), the pML-58 construct includes both the FBE element and the *yp1* promoter. Thus, the DNA sequences from nucleotide position −322 to −1225 are absent in pML-58, but present in pCR1.

We first examined the regulation of the pML-58 reporter gene by *dsx*. Our analysis showed that the regulatory sequences in pML-58 are sufficient to mediate regulation by *dsx*, consistent with previous findings (Coschigano and Wensink, 1993). DSX^F increases pML-58 activity 7-fold (Table 3, compare rows 1 and 2, P<0.001). This is only 1.4-fold less than the effect of DSX^F on the pCR1 activity in females (Table 1, 10-fold, compare rows 1 and 2, P<0.02), suggesting that pML-58 contains all of the DSX^F-responsive sequences. pML-58 also contains all of the DSX^M-responsive sequences since, when DSX^M is present, the *yp*-reporter on pML-58 is completely turned off, as is pCR1 (compare row 3 of Table 1 to row 3 of Table 3).

Table 3. *dsx*, but not *her*, regulates pML-58 expression

Row*	Genotypes†		*lacZ* activity (mean)‡	s.d.§	*n*¶
1	pML-58;	*dsx*/+	1.28×10^{-1}	7.30×10^{-4}	2
2	pML-58;	*dsx*	1.90×10^{-2}	3.30×10^{-3}	2
3	pML-58/Y;	*dsx*/+	5.41×10^{-4}	2.60×10^{-4}	2
4	pML-58/Y;	*dsx*	6.97×10^{-3}	6.40×10^{-4}	2
5	pML-58;	her^1/+	9.44×10^{-2}	1.39×10^{-2}	4
6	pML-58;	*her*	4.57×10^{-2}	9.40×10^{-3}	4
7	pML-58/Y;	her^1/+	3.71×10^{-4}	4.31×10^{-5}	4
8	pML-58/Y;	*her*	3.47×10^{-4}	7.14×10^{-5}	4

*Rows 1-4 and 5-8 form independent groups, respectively. Each group contains results from sibs. All flies were raised at 25°C and aged as adults at 25°C except that the flies in rows 5-8 were raised at 18°C and aged as adults at 25°C.

†The complete genotypes in rows 1-8 are as follows. The pML-58 reporter gene is X-linked. 1, pML-58/pML-58; *dsx* p^p/*MKRS*, *ry*. 2, pML-58/pML-58; *dsx* p^p/*dsx* p^p. 3, pML-58/ B^sY; *dsx* p^p/*MKRS*, *ry*. 4, pML-58/ B^sY; *dsx* p^p/*dsx* p^p. 5, pML-58/pML-58; *b* her^1/*SM1*; *ry*. 6, pML-58/pML-58; *b* her^1; *ry*. 7, pML-58/Y; *b* her^1/*SM1*; *ry*. 8, pML-58/Y; *b* her^1; *ry*.

‡-¶Same as in Table 2.

However, we found that the regulatory sequences of *yp1* and *yp2* in pML-58 are not sufficient to mediate regulation by *her*. Thus her^+ only increases the pML-58 activity 2-fold in females (Table 3, compare rows 5 and 6, P<0.001) while her^+ increases the pCR1 activity 11-fold in females (Table 1, compare rows 7 and 8, P<0.001). In addition, a previously described FBE-*hsp70* promoter-*lacZ* reporter gene that can mediate *dsx*'s regulation was not regulated by *her* (data not shown) (Garabedian et al., 1986). Thus, the DNA sequences from nucleotide position −322 to −1225 are necessary for *her* responsiveness (termed the *her* responsive region, HRR, hereafter). While pML-58 and pCR1 differ both with respect to the portions of the *yp1/2* regulatory region that they contain and in their promoters (pML-58 has the *yp1* promoter and pCR1 the *yp2* promoter), we believe that the difference in their responsiveness to *her* are due to the differences in the regulatory regions and not the differences in the promoters. In particular, we showed above by northern analysis that both the *yp1* and *yp2* genes responded identically to regulation by *her*. Moreover, the *yp1* and *yp2* promoters have been shown to be regulated sex-specifically, tissue-specifically and by *tra-2* and *dsx* in the same coordinated manner (Belote et al., 1985; Garabedian et al., 1985; Logan and Wensink, 1990; Tamura et al., 1985).

Since pML-58 responded well to *dsx* regulation but not to *her* regulation, a good responsiveness to *dsx* is not dependent on a good responsiveness to *her*, consistent with our earlier results that the DSX^F-dependent female-specific activation pathway is largely independent of the HER-dependent non-sex-specific activation pathway.

Since the HRR is necessary for about 5-fold responsiveness to *her*, loss of the HRR should lead to about 5-fold loss of non-sex-specific activation of the *yps*. This reasoning predicts that (1) in females lacking DSX^F, the *lacZ* activity of pML-58 should be about 5-fold less than that of pCR1, (2) in females with DSX^F, the *lacZ* activity of pML-58 should also be close to 5-fold less than that of pCR1, since the HER-dependent non-sex-specific activation is largely independent of DSX^F, and (3) in males lacking DSX^M, the *lacZ* activity of pML-58 should be about 5-fold less than that of pCR1. For all of these three comparisons, the appropriate control is the *lacZ* activity of pML-58 or pCR1 in wild-type male sibs, since neither reporter gene is expressed in males when DSX^M is present. Our data described below are in agreement with these predictions.

The pML-58 activity is 35-fold higher in *dsx/dsx* females than in their *dsx*/+ male sibs (Table 3, compare rows 2 and 3, P<0.02), while the pCR1 activity is 180-fold higher in *dsx/dsx* females than in their *dsx*/+ male sibs (Table 1, compare rows 2 and 3, P<0.01). Thus, as predicted, in females without DSX^F, the *lacZ* activity of pML-58 is about 5-fold less than that of pCR1.

The pML-58 activity is 240-fold higher in *dsx*/+ females than in their *dsx*/+ male sibs (Table 3, compare rows 1 and 3, P<0.001), while the pCR1 activity is 1800-fold higher in *dsx*/+ females than in their *dsx*/+ male sibs (Table 3, compare rows 1 and 3, P<0.001). Thus, in females with DSX^F, the *lacZ* activity of pML-58 is 7.5-fold less than that of pCR1. The 7.5-

Table 4. *dsx* and *her* regulate sexual differentiation via different mechanisms

		VT‡		DLAP§	LTRB¶		T5**		T6††		S6‡‡	
Row*	Genotypes†	Mean	s.d.	%	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
1	XX; *her/+ ; dsx/+*	27.7	1.9	100	5.3	0.5	37.8	4.0	77.3	6.6	18.6	2.2
2	XX; *her/+ ; dsx*	12.4	4.0	80	7.6	0.5	88.9	6.2	100.0	0.0	20.4	1.8
3	XX; *her; dsx/+*	10.5	3.2	30	7.3	1.2	84.0	4.8	99.6	1.6	18.3	2.3
4	XX; *her; dsx*	2.7	2.7	5	7.3	0.9	88.1	8.1	100.0	0.0	18.8	4.9
5	XY; *her/+ ; dsx/+*	0.0	0.0	0	11.4	1.4	92.1	3.7	100.0	0.0	0.1	0.4
6	XY; *her/+ ; dsx*	12.2	3.3	70	7.2	0.7	86.1	9.1	99.8	0.9	17.5	2.2
7	XY; *her; dsx/+*	0.0	0.0	0	10.1	1.2	92.2	3.7	100.0	0.0	6.2	2.0
8	XY; *her; dsx*	3.8	3.6	5	7.3	0.9	89.8	11.1	100.0	0.0	17.3	2.1

*Rows 1-8 contain results from sibs, thus data can be compared. All flies were raised at 23.5°C and aged as adults at 23.5°C. 20 flies were scored for each phenotype.

†Shown are progeny genotypes of parental cross: mother *w; b her^1/CyO; dsx p^p/TM6* × father *y; her^1 pr/CyO; dsx p^p/TM3.*

‡Vaginal teeth numbers.

§Shown are the percentage of flies scored that have fused intersexual dorsal-lateral anal plates. The dorsal anal plate of wild-type females is considered completely fused and the lateral anal plates of wild-type males are considered completely separated.

¶The numbers of the last transverse row of bristles of basal tarsus of forelegs. In wild-type males, it is the bristle number of a sex comb.

**The percentage width of the fifth tergite that are darkly pigmented.

††Same as ** except that the sixth tergite is measured.

‡‡The numbers of bristles on the sixth sternite.

fold reduction is close to the predicted 5-fold reduction. The slightly higher value than predicted could be due to the weak interaction between DSX^F and HER that we have detected using pCR1 (see above).

The pML-58 activity is 13-fold higher in *dsx/dsx* males than in their *dsx/+* male sibs (Table 3, compare rows 4 and 3, P<0.01), while pCR1 activity is 70-fold higher in *dsx/dsx* males than in their *dsx/+* male sibs (Table 1, compare rows 4 and 3, P<0.01). Thus, as predicted, in males without DSX^M, the *lacZ* activity of pML-58 is about 5-fold less than that of pCR1.

The reduced non-sex-specific activity of pML-58 could be due to the position effect of the chromosome insertion site of the transgene. However, this is not the case since three independent transformant lines of pML-58 showed similar low levels of the pML-58 activity in wild-type females (data not shown).

In conclusion, our data showed that the FBE is not sufficient to confer *her* responsiveness and the major *her* responsive element is located outside of FBE, in HRR (Fig. 2). Thus, HRR is necessary for the HER-dependent non-sex-specific activation of *yp1* and *yp2*.

Independent and dependent functioning of *dsx* and *her* in controlling female differentiation

The fact that *her* and *dsx* mutant females have similar external phenotypes (Pultz et al., 1994) raises the possibility that *dsx* and *her* may regulate other downstream target genes in a similar manner to how they regulate the *yp* genes. This predicts that the loss of *her* should masculinize *dsx* mutant XX flies and vice versa, since HER and DSX^F regulate the *yp* genes independently. In addition, the loss of *her* should also masculinize *dsx* mutant XY flies, since DSX^M inhibits *her*'s activation of the *yp* genes. To examine whether these predictions are true, we compared the phenotypes of five different external cuticular structures, which are sexually dimorphic in wild-type adult flies, among XX and XY sibs of the following four genotypes: (1) *her/+; dsx/+*, (2) *her/her; dsx/+*, (3) *her/+; dsx/dsx* and (4) *her/her; dsx/dsx.*

The first cuticular structure examined was the number of the vaginal teeth. Our results indicate that, in the precursor cells that give rise to vaginal teeth, *her* and *dsx* act independently as in the case of the regulation of the *yp* genes in fat body. Thus, there are on average 27.7 vaginal teeth on an XX; *her/+; dsx/+* fly and 0.0 on an XY; *her/+; dsx/+* fly (Table 4, column VT, row 1 and 5). The average number of vaginal teeth on an XX; *her/+; dsx/dsx* fly and on an XX; *her/her; dsx/+* fly is 12.4 and 10.5, respectively, indicating the intersexuality of these flies (Table 4, column VT, compare rows 1 and 2, P<0.0001; compare rows 1 and 3, P<0.0001). However, the number of vaginal teeth on an XX; *her/her; dsx/dsx* fly is 2.7, significantly less than on an XX; *her/+; dsx/dsx* fly (12.4) and on an XX; *her/her; dsx/+* fly (10.5) (Table 4, column VT, compare rows 4 and 2, P<0.0001; compare rows 4 and 3, P<0.0001). These results show that the loss of *her* masculinizes *dsx* mutant XX flies and vice versa, indicating that *her*$^+$ and *dsx*$^+$ can act in each other's absence in these cells.

her's function in vaginal teeth development is inhibited by DSX^M. The average number of vaginal teeth on an XY; *her/+; dsx/+* fly is 0.0, while on an XY; *her/+; dsx/dsx* fly, it is 12.2. However, in an XY; *her/her; dsx/dsx* fly, the number of vaginal teeth is 3.8, much less than that of XY; *her/+; dsx/dsx* flies (12.2) (Table 4, column VT, compare rows 6 and 8, P<0.0001). Thus the loss of *her* masculinizes *dsx* mutant XY flies, indicating that *her* is capable of promoting vaginal teeth development in XY flies if the inhibitory function of DSX^M is absent.

The second set of cuticular structures examined were the anal plates. The dorsal anal plate of females and the two lateral anal plates of males derive from the same precursor cells (Belote and Baker, 1982). In XX and XY intersex flies, there is a pair of anal plates located dorsolaterally to the anal opening and they are often fused at the dorsoanterior side. This pair of anal plates (referred to as DLAP hereafter) represents the intersexual differentiation of the precursor cells, and they are completely fused to form the dorsal anal plate in wild-type females and are completely separated to form the two lateral anal plates in wild-type males (Table 4, column DLAP, rows 1 and 5; Belote and Baker, 1982). Loss of *her* masculinizes *dsx* mutant XX flies and vice versa, since only 5% of DLAP in XX;

her/her; dsx/dsx are fused, while 80% and 30% of the DLAP in XX; *her/+; dsx/dsx* and XX; *her/her; dsx/+* flies are fused at the dorsoanterior side, respectively (Table 4, column DLAP, rows 1-4). Thus this result indicates that *her* and *dsx* can act independently in the DLAP precursor cells. In XY flies, DSX^M inhibits the *her*'s function in female-specific differentiation of DLAP, since only 5% of DLAP in XY; *her/her; dsx/dsx* flies are fused while 70% of DLAP in XY; *her/+; dsx/dsx* flies are fused, and 0% of DLAP in XY; *her/+* or *her/ her; dsx/+* flies are fused (Table 4, column DLAP, rows 5-8).

The remaining sexual dimorphic cuticular structures examined were (1) the number and morphology of the last (most distal) transverse row of bristles (LTRB) of the basitarsus (LTRB form sex combs in males), (2) the degree of pigmentation of tergite 5 (T5) and (3) the degree of pigmentation of tergite 6 (T6). In XX flies, the intersexual phenotypes of those structures are similar between *her* mutants and *dsx* mutants. The loss of *her* does not masculinize the *dsx* mutant XX flies in the LTRB precursor cells and vice versa (Table 4, column LTRB, compare rows 2-4). The same is true for T5 and T6 (Table 4, column T5, compare rows 2-4; column T6, compare rows 2-4). These results indicate that, in the LTRB, T5 and T6 precursor cells, DSX^F and HER are likely to act together in controlling sexual differentiation. When either HER or DSX^F is absent, the control is abolished. If this hypothesis is true, then in XY flies, loss of *her* should not masculinize *dsx* mutant flies in the LTRB, T5 and T6 precursor cells, since DSX^F is not present in those flies. This is indeed what was observed (Table 4, compare rows 6 and 8 in columns LTRB, T5 and T6).

In summary, our results indicate that, in the precursor cells of vaginal teeth and DLAP, HER controls downstream female-specific differentiation genes non-sex-specifically, and HER's functioning is independent of DSX^F in females and is inhibited by DSX^M in males, analogous to HER's regulation of the *yps* in fat body cells. However, our results also indicate that this is not the only mechanism by which *her* and *dsx* act. In the precursor cells of the LTRB of forelegs, T5 and T6, HER must function together with DSX^F.

The function of *dsx* and *her* in the development of Sternite 6

In addition to the cuticular structures described above, we also examined the number of the 6th sternite (S6) bristles on *dsx* mutant, *her* mutant, and *her; dsx* mutant XX and XY flies. The results indicate that (1) in XX flies, S6 differentiation follows a default pathway that is independent of *dsx* (DSX^F) and *her*, and (2) in XY flies, S6 differentiation is dependent on both *her* and *dsx* (DSX^M).

There are on average 18.6 S6 bristles on an XX; *her/+; dsx/+* fly, while there are virtually no S6 bristles on XY; *her/+; dsx/+* flies (Table 4, column S6, compare rows 1 and 5). In XX flies, loss of either *dsx* or *her*, or both, has no effect on the number of S6 bristles (Table 4, column S6, compare rows 1-5), indicating that *dsx* and *her* are not required for the S6 differentiation in XX flies. However, in XY flies the loss of *dsx* causes an increase of the number of S6 bristles to the female-specific level (Table 4, column S6, compare rows 1, 5 and 6), indicating that DSX^M suppresses bristle formation on S6 of XY flies. Consistent with the previous report (Pultz et al., 1994), HER, like DSX^M, is also required for the suppression of the bristle formation on S6 of XY flies. The loss of *her* in XY flies causes an increase in the average number of S6 bristles from virtually zero to 6.2 (Table 4, column S6, compare rows 5 and 7). This result indicates that a complete suppression of the S6 bristles requires both the DSX^M and HER functions (Table 4, column S6, compare rows 5 and 7). We think these results reflect the functioning of HER with DSX^M in male-specific sexual differentiation of S6. Note HER functions with DSX^F in female differentiation in some tissues and 93% of DSX^F sequences are also present in DSX^M (Burtis and Baker, 1989). An alternative explanation of *her*'s role in the suppression of the S6 bristles is that *her* may be involved in specifying the segmental identity of S6. We think this is unlikely, since there is no other evidence to indicate that *her* has homeotic functions and the increased pigmentation of T6 in *her* mutant females (Table 4, column T6, compare rows 1 and 3) is just the opposite of what would be expected if *her* is required to keep T6 (and S6) from adopting the segmental identity of a more anterior segment.

DISCUSSION

Dependent and independent functioning of *her* and *dsx*

Our studies of how the HER and DSX proteins control several different aspects of sexual differentiation have revealed that how these proteins are used varies depending on the particular sexual phenotype being examined. These findings thus provide new insights into the regulation of sexual differentiation.

Based on the sample of sexual phenotypes that we examined, the most prevalent way in which these proteins function is for HER and DSX^F to independently promote female differentiation. This is the manner in which they control the expression of the *yp* genes in the fat body where we have shown that there are two separate pathways for the activation of the *yps*. One is the female-specific activation of the *yps*, which is DSX^F-dependent. The other is the non-sex-specific activation of the *yps*, which is HER-dependent, DSX^F-independent and inhibited by DSX^M. Thus in wild-type females, both HER and DSX^F contribute independently to producing the high level of expression of the *yps* whereas, in wild-type males, the expression of the *yps* is prevented by the inhibitory function of DSX^M, overriding the activation function of HER. We have also shown that *her* and *dsx* control downstream target genes in a similar manner in the precursor cells that give rise to the vaginal teeth and dorsal anal plate of females and the lateral anal plates of males (DLAP).

However, in the precursor cells of the LTRB of the foreleg, T5, T6 and S6, *her* and *dsx* use different mechanisms to regulate sexual differentiation. It was suggested that DSX^F and DSX^M control, in opposite ways, a set of genes for the sex-specific differentiation of LTRB, T5 and T6 (Jursnich and Burtis, 1993). Our results suggest that for DSX^F to control target genes in these tissues, HER must be present, and vice versa. Thus the loss of either HER or DSX^F completely abolishes the regulatory pathway that involves both DSX^F and HER. Because of this strong dependent functioning of HER and DSX^F, HER is unable to promote female differentiation in males where DSX^F is absent. Thus unlike the fat body and the precursor cells of vaginal teeth and

DLAP, the male-specific differentiation of LTRB, T5 and T6 does not require the suppression of HER's function by DSX^M. Therefore, the loss of HER has no effect on the phenotypes of either XY; dsx^+ or XY; *dsx* flies. Although HER and DSX^F are similarly involved in the female-specific differentiation of LTRB, T5 and T6, DSX^M has different roles in the male-specific differentiation of those tissues. While in males, the loss of DSX^M leads to intersexual development of LTRB, and to some extent T5 as well (loss of pigmentation at the T5 anterior lateral margins), DSX^M is dispensable in the male-specific differentiation of T6 (see below). At the molecular level, DSX^M is likely to be responsible for upregulating the genes for pigment production in LTRB and T5, but in T6, DSX^M activity is not necessary as previously suggested (Jursnich and Burtis, 1993). Uniquely, DSX^M appears to act together with HER for the male-specific differentiation of S6, since DSX^M cannot completely suppress the S6 bristles of males when HER is absent.

Three mechanisms for the sexual dimorphism in *Drosophila*

Our analysis of sexual phenotypes of various tissues in the *her* and *dsx* single mutants and the *her*; *dsx* double mutants demonstrates that there are three ways by which sexual dimorphism is generated. The first utilizes DSX^M in males and does not require DSX^F in females. The second utilizes DSX^F in females and does not require DSX^M in males. The third utilizes both DSX^M in males and DSX^F in females. HER is involved in the last two modes of regulation, and likely also in at least some cases of the first mode of regulation.

On theoretical grounds, the most parsimonious way to generate differences between homologous tissues in the two sexes during evolution is to have a regulatory gene product present in the tissues of one sex and absent in the other sex, thus affecting the pre-existing non-sex-specific differentiation in one sex, but not in the other. For example, the default pathway for T6 is full pigmentation. The sexual dimorphism of T6 is solely due to the suppression of the T6 pigmentation by DSX^F in females, in collaboration with HER, and is irrespective of the presence or absence of DSX^M in males. Another example is the formation of S6 bristles. The default pathway is to form 18 bristles on S6. The sexual dimorphism of S6 is caused by the suppression of the bristle formation by DSX^M in males, likely in collaboration with HER, and is irrespective of DSX^F in females. However, in the presence of selective pressures on both sexes in evolution, one way to increase sexual dimorphism is to have female- and male-specific products of regulatory genes that each have active roles in modifying the effects of pre-existing non-sex-specific regulatory systems in opposite ways, thus generating dramatic sex-specific features. For instance, in the absence of DSX^F in females and DSX^M in males, the expression levels of the *yp* genes are equivalent between the two sexes due to the non-sex-specific control by HER. When females have DSX^F and males do not have DSX^M, there is a 30-fold difference between females and males in the expression levels of the *yp* genes, and when females do not have DSX^F and males have DSX^M, there is a 180-fold difference between females and males (see Table 1). However, a maximum difference (2000-fold) is observed only when DSX^F is present in females and DSX^M is present in males (see Table 1). The sexually dimorphic differentiation of the precursor cells of the vaginal teeth and DLAP is similarly controlled by HER and both DSX proteins. Thus, we may view *her* as part of a non-sex-specific regulatory system in those tissues, which is subject to the sex-specific modification by DSX^F and DSX^M.

On the molecular mechanism of the *her* and *dsx* functions

Besides providing the above general view of how sexual dimorphism is generated, our studies have also revealed some mechanistic details. We showed that, in females, HER and DSX^F activate the *yps* in a largely independent manner. This result is consistent with our finding that *her* and *dsx* act through distinct sequences of the regulatory region of the *yps*. Since HER is a zinc finger protein, it is likely to regulate the *yps* directly. Our finding that *her* activates the *yps* non-sex-specifically and *her* function is inhibited by DSX^M showed that the *her* and *dsx* genes must be expressed independently, and thus answered the question of whether *her* and *dsx* are expressed independently at the translational or post-translational levels, since previous studies showed that *her* and *dsx* do not regulate each other at the level of transcription or splicing (see Introduction).

That HER and DSX^F act largely independently in regulating the *yps* in the female fat body is in contrast to their strong interaction in controlling differentiation of the LTRB of the foreleg, and the pigmentation of T5 and T6. These differences could be due to different organizations of the regulatory elements of the differentiation genes being controlled in these tissues, or to differences between the arrays of other factors regulating these genes, which HER and DSX^F interact with. Alternatively, there may be a very low level of *her* activity present in the *her* mutant flies analyzed and that level is sufficient for DSX^F to function in the regulation of the *yps*, but not in the regulation of the genes being controlled in the LTRB of the foreleg or T5 and T6. Nevertheless, the finding that *her* and *dsx* act in different ways to control various aspects of sexual differentiation shows that the control of sexual differentiation is more complex than previously thought.

The fat-body-specific expression of the *yps* is not specified by *dsx* since, in *dsx* mutant flies of both sexes, the expression of the *yp*-reporter genes is still restricted to fat body cells, and this DSX^F-independent and non-sex-specific expression is repressed by DSX^M (H. L. and B. S. B., unpublished data; Coschigano and Wensink, 1993). Since HER controls the *yps* non-sex-specifically and independently of DSX^F, and HER function is repressed by DSX^M, the question arises as to whether HER is required for the fat-body-specific expression of the *yps*. We think HER is not necessary for the tissue-specific expression of the *yps*, since the FBE-*hsp70* promoter-*lacZ* and the pML-58 transgenes, which are not regulated by *her*, are still predominately, if not exclusively, expressed in fat body cells, and the loss of *her* does not affect fat-body-specific expression of the pCR1 transgene (H. L. and B. S. B., unpublished data). One possibility of how the fat-body specificity of the *yps* is controlled is by the unknown fat-body-specific factor(s) (referred to as the FBF hereafter) which is required independently of DSX^F and HER for *yp* expression. It has been proposed that the FBF may bind to a site (the bzip1 site) in the FBE that appears to be necessary

for the function of DSX^F (An et al., 1996; An and Wensink, 1995).

We have shown that the HRR is necessary for *her* to function. The question arises as to whether the HRR is sufficient for *her* function. Since the FBF, which likely binds to the FBE, is necessary for HER to activate the *yps*, as discussed above, the HRR is not likely to be sufficient for *yp* expression. Consistent with this notion, DNA fragments that contain HRR, but not the FBE, were previously shown to be unable to activate the expression of the *yps* in fat body (Garabedian et al., 1985, 1986).

Besides *dsx* and *her*, *ix* also acts in the *dsx* branch of the sex determination pathway. With respect to the regulation of the *yp* genes, we have observed that *ix* is also required for the transcriptional activation of the *yps* in females, but is not required for their repression in males (H. L., Garrett-Engele and B. S. B., unpublished data). Genetic studies indicate that *ix* interacts with *her* and with *dsx* in regulating the *yps* in females (H. L., Garrett-Engele and B. S. B., unpublished data), thus *ix* is likely to participate both in the HER-dependent non-sex-specific activation pathway and in the DSX^F-dependent female-specific activation pathway. One candidate DNA site in the FBE through which IX may function is the 'ref' site, which was shown to function synergistically with the DSX-binding site dsxA (An and Wensink, 1995).

In summary, we have shown that one set of female-specific terminal differentiation genes, the *yp* genes, is regulated by the *dsx*-dependent sex-specific pathway and the *her*-dependent non-sex-specific pathway. The integration of the two regulatory systems occurs at the level of transcriptional control. We have provided further evidence that some other unidentified sex-specific terminal differentiation genes are likely to be regulated similarly by *dsx* and *her*. However, this two-pathway mechanism does not apply to all of the sex-specific terminal differentiation genes. Tissue-specific factors and the organization of *dsx*- and *her*-binding sites in the regulatory sequences of terminal differentiation genes are likely to play important roles in determining which mechanism is used by *dsx* and *her* for the sexual differentiation of a particular tissue. Our results suggest that one way in which sexual differentiation is achieved is by sex-specific modification of the functions of non-sex-specific and tissue-specific regulatory genes.

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REFERENCES

An, W., Cho, S., Ishii, H. and Wensink, P. C. (1996). Sex-specific and non-sex-specific oligomerization domains in both of the doublesex transcription factors from Drosophila melanogaster. *Mol. Cell Biol.* **16,** 3106-11.

An, W. and Wensink, P. C. (1995). Integrating sex- and tissue-specific regulation within a single Drosophila enhancer. *Genes Dev.* **9,** 256-66.

Baker, B. S. and Ridge, K. (1980). Sex and the single cell: On the action of major loci affecting sex determination in *Drosophila melanogaster*. *Genetics* **94,** 383-423.

Belote, J. M. and Baker, B. S. (1982). Sex determination in Drosophila melanogaster: analysis of transformer-2, a sex-transforming locus. *Proc. Natl. Acad. Sci. USA* **79,** 1568-1572.

Belote, J. M., Handler, A. M., Wolfner, M. F., Livak, K. J. and Baker, B. S. (1985). Sex-specific regulation of yolk protein gene expression in Drosophila. *Cell* **40,** 339-48.

Boswell, R. E. and Mahowald, A. P. (1985). *tudor*, a gene required for assembly of the germ plasm in Drosophila melanogaster. *Cell* **50,** 97-104.

Bownes, M. (1994). The regulation of the *yolk protein* genes, a family of sex differentiation genes in *Drosophila melanogaster*. *BioEssays* **16,** 745-752.

Brennan, M. D., Weiner, A. J., Goralski, T. J. and Mahowald, A. P. (1982). The follicle cells are a major site of vitellogenin synthesis in Drosophila melanogaster. *Dev. Biol.* **89,** 225-236.

Burtis, K. C. (1993). The regulation of sex determination and sexually dimorphic differentiation in Drosophila. *Curr. Opin. Cell. Biol.* **5,** 1006-1014.

Burtis, K. C. and Baker, B. S. (1989). Drosophila *doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. *Cell* **56,** 997-1010.

Burtis, K. C., Coschigano, K. T., Baker, B. S. and Wensink, P. C. (1991). The *doublesex* proteins of *Drosophila melanogaster* bind directly to a sex-specific *yolk protein* gene enhancer. *EMBO J.* **10,** 2577-2582.

Burtis, K. C. and Wolfner, M. F. (1992). The view from the bottom: Sex-specific traits and their control in *Drosophila*. *Semin. Dev. Biol.* **3,** 331-340.

Chase, B. A. and Baker, B. S. (1995). A genetic analysis of *intersex*, a gene regulating sexual differentiation in Drosophila melanogaster females. *Genetics* **139,** 1649-1661.

Cline, T. W. and Meyer, B. J. (1996). Vive la différence: males vs females in flies vs worms. *Annu. Rev. Genet.* **30,** 637-702.

Coschigano, K. T. and Wensink, P. C. (1993). Sex-specific transcriptional regulation by the male and female doublesex proteins of Drosophila. *Genes Dev.* **7,** 42-54.

Erdman, S. E., Chen, H. and Burtis, K. C. (1996). Functional and genetic characterization of the oligomerization and DNA binding properties of the *Drosophila doublesex* proteins. *Genetics* **144,** 1639-1652.

Gailey, D. A., Taylor, B. J. and Hall, J. C. (1991). Elements of the *fruitless* locus regulate development of the muscle of Lawrence, a male-specific structure in the abdomen of *Drosophila melanogaster* adults. *Development* **113,** 879-890.

Garabedian, M. J., Hung, M. C. and Wensink, P. C. (1985). Independent control elements that determine yolk protein gene expression in alternative Drosophila tissues. *Proc. Natl. Acad. Sci. USA* **82,** 1396-400.

Garabedian, M. J., Shepherd, B. M. and Wensink, P. C. (1986). A tissue-specific transcription enhancer from the Drosophila yolk protein 1 gene. *Cell* **45,** 859-867.

Hall, J. C. (1994). The mating of a fly. *Science* **264,** 1702-1714.

Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (1990). *PCR protocols: a guide to methods and applications*. San Diego: Academic Press Inc.

Ito, H., Fujitani, K., Usui, K., Shimizu-Nishikawa, K., Tanaka, K. and Yamamoto, K. (1996). Sexual orientation in *Drosophila* is altered by the *satori* mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain. *Proc. Natl. Acad. Sci. USA* **93,** 9687-9692.

Jursnich, V. A. and Burtis, K. C. (1993). A positive role in differentiation for the male *doublesex* protein of *Drosophila*. *Dev. Biol.* **155,** 235-249.

Lawrence, P. A. and Johnston, P. (1986). The muscle pattern of a segment of *Drosophila* may be determined by neurons and not by contributing myoblasts. *Cell* **45,** 505-513.

Li, H. and Baker, B. S. (1998). *her*, a gene required for sexual differentiation in *Drosophila*, encodes a zinc finger protein with characteristics of ZFY-like proteins and is expressed independently of the sex determination hierarchy. *Development* **125,** 225-235.

Lindsley, D. L. and Zimm, G. (1992). *The Genome of Drosophila melanogaster.* New York: Academic Press.

Logan, S. K. and Wensink, P. C. (1990). Ovarian follicle cell enhancers from the Drosophila yolk protein genes: different segments of one enhancer have

different cell-type specificities that interact to give normal expression. *Genes Dev.* **4,** 613-623.

Lossky, M. and Wensink, P. C. (1995). Regulation of Drosophila yolk protein genes by an ovary-specific GATA factor. *Mol. Cell. Biol.* **15,** 6943-6952.

McKeown, M. (1992). Alternative messenger-RNA splicing. *Ann. Rev. Cell. Biol.* **8,** 133-155.

McKeown, M. and Madigan, S. J. (1992). Sex determination and differentiation in invertebrates: *Drosophila* and *Caenorhabditis elegans*. *Curr. Opin. Cell. Biol.* **4,** 948-954.

Ota, T., Fukunaga, A., Kawabe, M. and Oishi, K. (1981). Interactions between sex-transformation mutants of Drosophila melanogaster. I. Hemolymph vitellogenins and gonad morphology. *Genetics* **99,** 429-41.

Parkhurst, S. M. and Meneely, P. M. (1994). Sex determination and dosage compensation: lessons from flies and worms. *Science* **264,** 924-932.

Postlethwait, J. H., Laugé, G. and Handler, A. M. (1980). Yolk protein synthesis in ovariectomized and genetically agametic *[X^{87}] Drosophila melanogaster*. *Gen. Comp. Endocrinol.* **40,** 385-390.

Pultz, M. A. and Baker, B. S. (1995). The dual role of *hermaphrodite* in the *Drosophila* sex determination regulatory hierarchy. *Development* **121,** 99-111.

Pultz, M. A., Carson, G. S. and Baker, B. S. (1994). A genetic analysis of *hermaphrodite*, a pleiotropic sex determination gene in *Drosophila melanogaster*. *Genetics* **136,** 195-207.

Ryner, L., Goodwin, S. F., Castrillon, D. H., Anand, A., Villella, A., Baker, B. S., Hall, J. C., Taylor, B. J. and Wassserman, S. A. (1996). Control of male sexual behavior and sexual orientation in *Drosophila* by the *fruitless* gene. *Cell* **87,** 1079-1089.

Tamura, T., Kunert, C. and Postlethwait, J. (1985). Sex- and cell-specific regulation of yolk polypeptide genes introduced into Drosophila by P-element-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* **82,** 7000-7004.

Taylor, B. J. and Truman, J. W. (1992). Commitment of abdominal neuroblasts in *Drosophila* to a male or female fate is dependent on genes of the sex-determining hierarchy. *Development* **114,** 625-642.

Taylor, B. J., Villella, A., Ryner, L. C., Baker, B. S. and Hall, J. C. (1994). Behavioral and neurobiological implications of sex-determining factors in *Drosophila*. *Dev. Genet.* **15,** 275-96.

Villella, A. and Hall, J. C. (1996). Courtship anomalies caused by *doublesex* mutations in *Drosophila melanogaster*. *Genetics* **143,** 331-344.