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The dual role of *hermaphrodite* in the *Drosophila* sex determination regulatory hierarchy

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SUMMARY

The *hermaphrodite (her)* locus has both maternal and zygotic functions required for normal female development in *Drosophila.* Maternal *her* function is needed for the viability of female offspring, while zygotic *her* function is needed for female sexual differentiation. Here we focus on understanding how *her* fits into the sex determination regulatory hierarchy. Maternal *her* function is needed early in the hierarchy: genetic interactions of *her* with the *sisterless* genes (*sis-a* and *sis-b),* with function-specific *Sex-lethal (Sxl)* alleles and with the constitutive allele $Sxl^{M\#1}$ suggest that maternal *her* function is needed for *Sxl* initiation. When mothers are defective for *her* function, their daughters fail to activate a reporter gene for the *Sxl* early promoter and are deficient in *Sxl* protein expression. Dosage compensation is misregulated in the moribund daughters: some salivary gland cells show binding of the *maleless (mle)* dosage compensation regulatory protein to the X chromosome, a binding pattern normally seen only in males. Thus maternal *her* function is needed early in the hierarchy as a positive regulator of *Sxl,* and the maternal effects of *her* on female viability probably reflect *Sxl*'s role in regulating dosage compensation. In contrast to *her*'s maternal function, *her*'s zygotic function in sex determination acts at the end of the hierarchy. This zygotic effect is not rescued by constitutive *Sxl* expression, nor by constitutive *transformer (tra)* expression. Moreover, the expression of *doublesex (dsx)* transcripts appears normal in *her* mutant females. We conclude that the maternal and zygotic functions of *her* are needed at two distinctly different levels of the sex determination regulatory hierarchy.

Key words: *Drosophila*, sex determination, *hermaphrodite*

INTRODUCTION

Sex determination and dosage compensation in *Drosophila* are controlled by an elaborate regulatory hierarchy (for reviews, see Baker, 1989; Slee and Bownes, 1990; Steinman-Zwicky et al., 1990; Belote, 1992; Burtis and Wolfner, 1992; McKeown and Madigan, 1992; Cline, 1993; Cronmiller and Salz, 1993; Parkurst and Meneely, 1994), and new components of this hierarchy continue to be discovered. Here we focus on the pleiotropic sex determination gene *hermaphrodite (her),* described recently (Pultz et al., 1994). Wild-type *her* function must be supplied both maternally and zygotically for normal female development – the maternal function is needed for female viability and the zygotic function is needed for female sexual differentiation. To understand how *her* fits into the sex determination hierarchy, we have examined the interactions of *her* mutations with many of the known sex determination and dosage compensation genes. We review here briefly those aspects of this hierarchy that are relevant to this study.

In *Drosophila,* sex is determined by the ratio of X chromosomes to autosome sets, which establishes the activity state of *Sxl. Sxl* activity is initiated and maintained only in females. In the female soma, *Sxl* controls sexual differentiation and is also responsible for limiting the level of X-chromosome expression (i.e. preventing dosage compensation). *Sxl* is also active and necessary in the female germline (reviewed in Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992), but here we will focus only on the soma. The control of *Sxl* expression depends on positive and negative regulators, supplied maternally and zygotically (for recent reviews, see Cline, 1993; Cronmiller and Salz, 1993; Parkhurst and Meneely, 1994). *Sxl* initiation is transcriptionally controlled and *Sxl* function is then maintained through regulation at the level of splicing. *Sxl* initiation depends on a female-specific 'early' promoter, which generates protein-coding mRNAs (Keyes et al., 1992). Later, *Sxl* is transcribed in both sexes from a sex non-specific 'late' promoter; but only females synthesize *Sxl* protein due to sex-specific regulation of RNA splicing (Bell et al., 1988; Salz et al., 1989; Bopp et al., 1991). The female-specific splice choice is regulated by *Sxl* protein in a positive autoregulatory feedback loop initiated by products of the early *Sxl* promoter (Cline, 1984; Bell et al., 1991; Keyes et al., 1992).

Downstream of *Sxl,* somatic sexual differentiation is controlled by a cascade of regulated splicing, such that a signal for

female differentiation is transmitted from *Sxl* via *transformer (tra)* to *doublesex* (*dsx*) (for reviews, see Baker, 1989; Steinmann-Zwicky et al, 1990; Belote, 1992; Mattox et al., 1992; McKeown and Madigan, 1992). First, *Sxl* regulates *tra* at the level of RNA splicing, generating a female-specific protein-coding *tra* mRNA. Then *tra* protein collaborates with *transformer-2 (tra-2)* protein to regulate *dsx* at the level of RNA splicing. This generates a female-specific *dsx* mRNA encoding a female-specific version of the *dsx* protein. (In males, by default, an alternatively spliced male-specific *dsx* mRNA is produced, encoding a male-specific version of the *dsx* protein). Function of *intersex (ix)* is also needed for female sexual differentiation, though *ix* is not needed for the sex-specific splicing of *dsx* (Nagoshi et al, 1988). Therefore *ix* and *dsx* may collaborate in controlling female-specific gene expression.

At the point of *dsx* function, the control of gene expression is passed from the level of splicing back to the level of transcription. For example, the *dsx* proteins are zinc finger related transcription factors that bind to sex-specific enhancer sequences in the yolk protein gene (Burtis et al., 1991; Coschigano and Wensink, 1993; Erdman and Burtis, 1993). Most sexually dimorphic aspects of differentiation are under *dsx* control, directly or indirectly (reviewed in Burtis and Wolfner, 1992). However, some aspects of nervous system development (including behavior) have recently been found to be regulated only by *tra*, not by *dsx* or *ix* (Lawrence and Johnston, 1986; Taylor, 1992; reviewed in Taylor et al., 1994; Hall, 1994). Thus there is at least one previously unrecognized branch within that part of the regulatory hierarchy that controls somatic sexual differentiation.

Another major branch of the sex determination regulatory hierarchy controls the regulation of X-linked gene expression, or dosage compensation. In *Drosophila* females, both X chromosomes are transcribed at a basal level in each cell, and dosage compensation occurs via the hypertranscription of the single X chromosome in males. Dosage compensation in males depends on the functions of the *male-specific-lethal* genes (for reviews, see Lucchesi and Manning, 1987; Kuroda et al., 1993; Baker et al., 1994; Gorman and Baker, 1994). Proper regulation of dosage compensation is essential for viability both in females and in males, hence the sex-specific lethal phenotypes of genes involved in this process. The *mle*, *msl-1* and *msl-3* proteins bind specifically to hundreds of sites along the X chromosome in males but not in females (Kuroda et al., 1991; Palmer et al., 1993; Gorman et al, in press) and are thought to directly mediate dosage compensation. In females, *Sxl* is responsible for preventing X-chromosome hypertranscription (Lucchesi and Skripsky, 1981; Gergen, 1987), and for preventing the *mle*, *msl-1* and *msl-3* proteins from binding, inappropriately, to the X chromosome (Gorman et al., 1993; Palmer et al., 1994; Gorman et al., in press).

Here we focus on delineating the role of *hermaphrodite* (*her*) in the sex determination regulatory hierarchy. Based on the phenotypic effects of four loss-of-function alleles, *her* has multiple functions (Pultz et al., 1994). Zygotic *her* function is important for sexual differentiation: intersexual phenotypes are seen in chromosomal females that are homozygous for loss-of-function *her* alleles. Zygotic *her* function also appears to be needed for sexual differentiation in males, though existing *her* alleles affect male-specific development only weakly. Maternal *her* function is required sex-specifically for the viability of daughters (assayed at 25°C). Finally, wild-type *her* function is also required maternally and zygotically for viability in both sexes (assayed at 29°C). To understand the female-specific maternal and zygotic roles of *her*, we have examined interactions of *her* mutations with many of the regulatory genes described above. We find that *her* must function at two different levels of the sex determination regulatory hierarchy.

MATERIALS AND METHODS

The *her* alleles

A full description of the *her* alleles is given in Pultz et al. (1994). All alleles fail to complement for sex-specific maternal effects; her^1, her^2 and her^3 fail to complement inter se for zygotic effects on sexual differentiation. All alleles are temperature-sensitive and all behave as hypomorphs over a deficiency for the *her* locus. The *her* locus is covered by the duplication *Dp(2;3)* osp^3, *Ki* her^+.

Maternal effects

To test for viability and regulatory gene expression in progeny, *her* experimental mothers and sibling her^+ control mothers were generated by crossing *her*/balancer females to *her*/balancer; *Dp(2;3)* osp^3, *Ki* her^+/+ males. This generates experimental *her*/*her* mothers and sibling control mothers of the genotype *her*/*her*; *Dp(2;3)* osp^3, *Ki* her^+ which differ from the experimental mothers only by the presence of the duplication-bearing chromosome. Mothers were raised at 18°C and brooded at 25°C. For further detail concerning procedures and precautions in *her* maternal effect experiments, see the Materials and Methods of Pultz et al. (1994).

Genotypes and crosses

For information on marker genes and balancer chromosomes, see Lindsley and Zimm (1992). For all crosses, *Dp(*her^+*) = Dp(2;3)*osp^3, *Ki* her^+. Full genotypes are described below the tables. Additional details for Table 3A are given below.

Table 3A

y w^a $Sxl^{M\#1}$ *sn*/*cm; b* her^1/*SM1, Cy* mothers × *cm* ; *b* her^1/*SM1, Cy* fathers. The combination w^a *cm* gives a whiter eye color than w^a, making it possible to score *cm* in the presence of w^a in males. Two classes of her^1 individuals were recovered: members of one class were intersexual and members of the other class were 'male', with a few sixth sternite bristles. The intersexes were assumed to be XX and the flies with male-like morphology were assumed to be XY. As expected, the her^1 females were all *cm* or cm^+, whereas the *her* males included recombinants for X chromosome markers: *y*, a few *sn*, and w^a *cm*.

Expression of the *Sxl* early promoter

Embryos were fixed and stained using standard *Drosophila* fixation and antibody staining procedures (see below). An anti-beta-galactosidase monoclonal antibody was used (Sigma, at 1:750), followed by a horseradish peroxidase-conjugated secondary antibody (Jackson Labs, at 1:750), staining was with DAB and 0.1 % nickel chloride. Fathers were homozygous for the *lacZ* reporter gene construct of Keyes et al. (1992) on the second chromosome. This construct fuses 3 kb, containing the *Sxl* early promoter, upstream of the putative translation start site for *lacZ*. In wild-type *Drosophila*, the early *Sxl* promoter lies within an intron of the late *Sxl* transcript, and is separated from the late promoter by approximately 5 kb (Keyes et al, 1992). Mothers in this experiment were (a) control: *b* $her^{l(2)mat}$/her^1 *pr; Dp(2;3)*osp^3, *Ki* her^+, or (b) experimental: *b* $her^{l(2)mat}$/her^1 *pr.*

Survival for siblings of these embryos was (a) control: 270 females, 268 males (b) experimental: 6 females, 166 males. Embryos from the original homozygous *lacZ* reporter gene strain were also examined for comparison. These were similar to the above controls in the degree of difference between darkly stained and unstained embryos, though (as expected) the darkest staining was more intense than for embryos with only a single copy of the reporter gene.

Anti-SXL staining of embryos

Embryos were prepared and stained using the procedure described in the original characterization of the anti-SXL antibody by Bopp et al. (1991), except that goat serum was added to 5% during primary and secondary antibody incubations, and for 30 minutes preceding these incubations. Embryos were photographed using Nomarski optics. The full genotype of the cross for this experiment was $her^{l(2)mat}/her^{3}$ *pr* mothers × Canton S fathers (experimental) or $her^{l(2)mat}/her^{3}$ *pr; Dp(2;3)osp*^{3}*, Ki* her^{+} mothers × Canton S fathers (control). For mutant mothers, approximately 20-30% of the expected number of daughters survived to adulthood in this experiment; for control mothers, survival of daughters was approximately equal to survival of sons. Numbers of staining embryos: (1) control mothers – 100 fully stained embryos, 11 partially stained embryos, 108 unstained embryos; (2) mutant mothers – 104 partially stained embryos, 108 unstained embryos.

Anti-MLE staining of polytene chromosomes

Chromosomes were stained for binding of *mle* protein using the rabbit polyclonal anti-MLE antibodies of Kuroda et al. (1991) and the procedure of Gorman et al. (1993). The full genotype of the cross for this experiment was *y*/*y; her*^{1}*px sp*/$her^{l(2)mat}$ mothers × Canton S fathers (experimental) or *y*/*y; her*^{1}*px sp*/$her^{l(2)mat}$ *; Dp(2;3)osp*^{3}*, Ki* her^{+} mothers × Canton S fathers (controls). Each larva was sexed both by gonadal morphology and by scoring mouthparts for *y*. Among the progeny of her^{-} mothers in this experiment, the ratio of surviving daughters to surviving sons was approximately 1:4. The MLE-binding pattern in sons of her^{-} mothers appeared to be the same as in sons of her^{+} control mothers.

Northern blot analysis

Northern blots were performed as in Burtis and Baker (1989). Briefly, total RNA was prepared from 3-4 adults, separated by electrophoresis, blotted and incubated with a single-stranded DNA probe made with sequences common to both the male-specific and the female-specific forms of *dsx* transcripts. The female and male wild-type controls (lanes A and B) are as modified from Burtis and Baker (1989). Reprobing of the blot with ribosomal protein 49 (O'Connell and Rosbash, 1984) was with a double-stranded DNA probe prepared by priming with random oligonucleotides. Full genotypes for this experiment are her^{1} *or*/her^{1} *or, her1 or*/*SM1* and Canton S. The her^{1}/+ control flies and her^{1} intersexual flies and her^{1}/*SM1* control flies were raised at 25°C and held at 29°C for 2-3 days before preparation of RNA; wild-type flies were cultured at 25°C.

RESULTS

We have analyzed the maternal and zygotic functions of *hermaphrodite* separately to ascertain the role that each of them plays in sex determination and dosage compensation. Our dissection of the various aspects of *her* function has taken advantage of the temperature sensitivity of the *her* alleles (Pultz et al., 1994). For example, her^{1}/her^{2} females develop as sterile intersexes at semi-restrictive or restrictive temperatures (25°C or 29°C, respectively); but when raised at the permissive temperature of 18°C they are sexually normal and fertile, and can then be cultured at 25°C to assay sex-specific maternal effects. We have also taken advantage of the $her^{l(2)mat}$ allele (Redfield, 1926), which is severely defective in maternal *her* functions but comparable to wild-type in zygotic *her* functions (Pultz et al., 1994).

Maternal effects of *her* on female viability

The maternal function of *her* is needed for the viability of female progeny; at 25°C, the daughters of *her* mutant mothers survive only about 5-50% as well as sons (Redfield, 1926; Pultz et al., 1994). To determine the role of the maternal *her* function in the initial steps of sex determination, we examined the genetic interactions of *her* with the *sisterless* genes (positive regulators of *Sxl* initiation) and with specific loss-of-function and gain-of-function *Sxl* alleles. We also examined whether defective maternal *her* function affects the expression and/or distribution of *Sxl* and *mle* proteins.

Genetic interactions with the *sisterless* genes

The *sisterless* genes (*sis-a*, *sis-b* and *sis-c*) are X-linked positive regulators of *Sxl* that function zygotically during the first few hours of development (Cline, 1986, 1988; Torres and Sánchez, 1991; Erikson and Cline, 1991; reviewed in Cline 1993) and fulfill the requirements for X chromosome counting elements. Most importantly, there are reciprocal phenotypic effects of altered *sisterless* gene function in males and females. For example, loss of *sis-a* or *sis-b* function is deleterious to female viability, due to inadequate activation of *Sxl*. Reciprocally, a duplication that includes both *sis-*a^{+} and *sis-*b^{+} is deleterious to male viability, due to inappropriate activation of *Sxl* (Cline, 1988; Erikson and Cline, 1993). These findings lead to the prediction that if maternal *her* function is necessary for the zygotic function of the *sisterless* genes, the impaired maternal function of *her* should exacerbate the effects of *sisterless* mutations in females and ameliorate the effects of extra wild-type copies of *sisterless* genes in males.

Table 1A shows that daughters of her^{-} mothers are severely affected by the presence of a *sis-a* point mutation, even when they also carry one *sis-*a^{+} allele. In these experiments, we compared the relative viabilities (daughters/sons) for different *sis-a* and maternal *her* genotypes. By this measure, the *sis-a*/*sis-*a^{+} daughters of her^{-} mothers were at least 50-fold less viable than *sis-*a^{+}/*sis-*a^{+} daughters from crosses with the same maternal genotypes. Moreover, even the *sis-a*/*sis-*a^{+} daughters of mothers with one her^{+} allele were not fully viable compared to their *sis-*a^{+}/*sis-*a^{+} counterparts. These results suggest that the wild-type maternal function of *her* may be participating in the same process as *sis-*a^{+} during female development.

Table 1B shows a reciprocal interaction between maternal *her* function and zygotic *sis* gene function in males. Here, defective maternal *her* function alleviates the semi-lethality of a *sisterless* duplication (containing *sis-a* plus *sis-b*) by approximately ten-fold. Thus it appears that defective maternal *her* function limits $sisterless^{+}$ function in both sexes.

The relationship between maternal *her* function and the *sis* genes can be probed further by considering the relative viabilities of female progeny with various doses of *sis* genes (Table 1B). One class of daughters are euploid for *sis-*a^{+} and have one extra copy of *sis-*b^{+}. When maternal *her* function is defective, these daughters are (at least) as viable as sons with euploid *sis* gene dosage. Thus, extra zygotic *sis-*b^{+} dosage can apparently overcome the usual female morbidity associated

Table 1. Maternal effects of *her*: interactions with *sisterless* genes

(A) Survival of *sis-a*/*sis-a*$^+$ daughters

	Paternal genotype			
	sis-a			*sis-a*$^+$
	daughters *sis-a*/*sis-a*$^+$	sons *sis-a*$^+$	Ratio (daughters:sons)	Ratios (range)† (daughters:sons)
Maternal genotype				
Experimentals				
her^1/*her*$^{l(2)mat}$	2	1673	**0.001**	0.06 - 0.23
her^1/*her*^{2}	1	688	**0.002**	0.14 - 0.50
Controls				
her^1/*her*$^{l(2)mat}$; Dp(*her*$^+$)/+	216	351	0.62	0.93 - 1.01
her^1/*her*^{2}; Dp(*her*$^+$)/+	287	376	0.68	0.91 - 0.93

The *her*$^-$ mothers (or control mothers) were crossed to *sis-a* fathers or to control *sis-a*$^+$ wild-type fathers, such that daughters all carry the (X-linked) *sis-a*$^+$ allele from their mothers and the *sis-a* or *sis-a*$^+$ allele inherited from their fathers. Sons are all *sis-a*$^+$. All crosses, 25°C.

†Ranges were assembled from data in Pultz et al. (1994) and from crosses using wild-type Crimea fathers (M. A. Pultz, unpublished).

Full genotypes: *sis-a = y sis-a* (Cline, 1986). *her*^{1}/*her*^{2} = *her*^{1} *or*/*SM1, her*^{2} *Cy. her*^{1}/*her*$^{l(2)mat}$ = *her*^{1} *pr*/*b her*$^{l(2)mat}$ (for *sis-a* crosses), *her*^{1} *or*/*b her*$^{l(2)mat}$ and *her*^{1} *pr*/*her*$^{l(2)mat}$ (for *sis-a*$^+$ crosses).

(B) Survival of sons with a duplication of *sis-a*$^+$ plus *sis-b*$^+$

	Daughters w/Dp *sis-a*$^+$: 2 copies *sis-b*$^+$: 3 copies	Daughters w/out *sis-a*$^+$: 1 copy *sis-b*$^+$: 2 copies	Sons with Dp	Sons w/out Dp	Ratio of sons with: sons w/out
Maternal genotype					
Experimentals					
her^1/*her*$^{l(2)mat}$	354	0	131	224	**0.58**
her^1/*her*^{2}	149	24	94	150	**0.63**
her^3/*her*$^{l(2)mat}$	316	59	203	316	**0.64**
Controls					
her^1/*her*$^{l(2)mat}$; Dp(*her*$^+$)/+	203	196	12	245	**0.05**
her^1/*her*^{2}; Dp(*her*$^+$)/+	447	445	16	488	**0.03**
her^3/*her*$^{l(2)mat}$; Dp(*her*$^+$)/+	375	355	36	519	**0.07**
her$^{l(2)mat}$/Gla	480	561	56	541	**0.10**
her$^{l(2)mat}$ /Gla; Dp(*her*$^+$)/+	403	404	31	434	**0.07**

The *sis*-duplication, on the second chromosome, was introduced from Df(*sis-a*$^-$); Dp(*sis-a*$^+$,*sis-b*$^+$)/+ fathers. Sons all inherit one X chromosome (with *sis-a*$^+$, *sis-b*$^+$) from their mothers, and half of the sons inherit the duplication. Daughters all inherit Df(*sis-a*$^-$) from their fathers, and half of the daughters inherit the duplication.

Experimental crosses: *her*$^-$ females × Df*(sis-a*$^-$*)* ; Dp*(sis-a*$^+$*, sis-b*$^+$*)* males, 25°C.

Control crosses: *her*$^-$; Dp*(her*$^+$*)* /+ females × Df*(sis-a*$^-$*)* ; Dp*(sis-a*$^+$*, sis-b*$^+$*)* males, 25°C.

Genotypes of progeny for *sis-a* and *sis-b*

Sons: with Dp: *sis-a*$^+$, *sis-b*$^+$; Dp (*sis-a*$^+$, *sis-b*$^+$)
w/out Dp: *sis-a*$^+$, *sis-b*$^+$

Daughters: with Dp: *sis-a*$^+$, *sis-b*$^+$/ Df(*sis-a*$^-$), *sis-b*$^+$; Dp (*sis-a*$^+$, *sis-b*$^+$)
w/out Dp: *sis-a*$^+$, *sis-b*$^+$/ Df(*sis-a*$^-$), *sis-b*$^+$

Full genotypes: *her*^{1}/ *her*$^{l(2)mat}$ = *her*^{1} *or* /*b her*$^{l(2)mat}$. *her*^{1}/ *her*^{2} = *her*^{1} *pr*/ *SM1, her*^{2} *Cy. Dp (sis-a*$^+$,*sis-b*$^+$*) = X*^{d} *2*^{p} *T(1;2)Hw*^{bap}, *y*$^+$*Hw*^{bap}*sis-b*$^+$ & *Dp(1;2)v*^{65b}, *v*^{m}*sis-a*$^+$*m*$^+$,*bw* (designated also as *DD(2)Ha* by Cline, 1988). *Df(sis-a*$^-$*)= Df(1)N71, sis-a*$^-$.

with defective *her* maternal function (Control Ratio, Table 1A), consistent with the view that *her* and the *sis* genes may participate in the same process. A second class of daughters (*Df(sis-a*$^-$*)*/*sis-a*$^+$) have euploid *sis-b*$^+$ dosage but only a single dose of *sis-a*$^+$. The relative viabilities for this class are only partially consistent with expectations raised by experiments with the *sis-a* point mutation above (Table 1A). With the maternal genotype *her*^{1}/*her*$^{l(2)mat}$, the *Df(sis-a*$^-$*)*/*sis-a*$^+$ daughters fail to survive, consistent with the inability of *sis-a*/*sis-a*$^+$ daughters to survive. However, with the maternal genotypes *her*^{1}/*her*^{2} or *her*^{3}/*her*$^{l(2)mat}$, the *Df(sis-a*$^-$*)*/*sis-a*$^+$ daughters survive approximately as well as would be expected for *sis-a*$^+$/*sis-a*$^+$ daughters (Table 1A and Pultz et al., 1994). Of the three *her* genotypes tested, *her*^{1}/*her*$^{l(2)mat}$ is the most severely defective in maternal *her* functions (Pultz et al., 1994), consistent with the above differences among maternal genotypes. Comparing zygotic genotypes, daughters appear to be more disadvantaged when heterozygous for a *sis-a* point mutation than when lacking one copy of *sis-a*$^+$ altogether, though such differences could also be ascribed to differences in genetic background.

The experiments above suggest that maternal *her* function participates in the same process as *sisterless* function. When daughters lack maternal *her* function, their prospects are improved by a duplication for *sis-b*$^+$, but worsened by a single copy of a *sis-a* mutation. If males are burdened with extra wild-type *sis* genes, their chances of surviving increase when maternal *her* function is defective.

Genetic interactions with *Sxl*

The preceding data suggest that maternal *her* products function early in the sex determination hierarchy, with other positive

Table 2. Maternal effects of *her*: interactions with *Sxl*

(A) Constitutive *Sxl* expression

	Female progeny genotype†		Male progeny genotype†	
	$Sxl^{M\#1}$	Sxl^{+}	$Sxl^{M\#1}$	Sxl^{+}
Experimental her^{-} mothers:	436	107	0	238
Control her^{+} mothers:	514	439	0	493

Experimental cross: $Sxl^{M\#1}/cm; her^{1}/her^{l(2)mat}$ females × *cm* males, 25°C
Control cross: $Sxl^{M\#1}/cm; her^{1}/ her^{l(2)mat}$; *Dp* $(her^{+})/+$ females × *cm* males, 25°C

†*Sxl* and *cm* are very closely linked, (0 recombinants in >10,000 scored - Cline, 1978), so the genotype for *Sxl* is inferred from the genotype for *cm*.

Full genotypes: $Sxl^{M\#1}$ = *y* w^{a} $Sxl^{M\#1}$*sn*. her^{1} = her^{1} *pr*. $her^{l(2)mat}$ = *b* $her^{l(2)mat}$. Dp (her^{+}) = Dp(2;3)*osp*^{3}, *Ki*. The combination w^{a} *cm* gives a whiter eye color than w^{a}, making it possible to score *cm* in the presence of w^{a} in males.

(B) Function-specific *Sxl* alleles

	Paternal genotype			
	Ratio (daughters:sons)		Ratio, daughters:sons	
	I 'initiation-defective' Sxl^{f9}	II 'maintenance-defective' Sxl^{fLS}	III* null $Sxl^{f\#1}$	IV* wild type (range) Sxl^{+}
Maternal genotype				
Experimentals				
$her^{1}/her^{l(2)mat}$	**<0.002** (0:466)	**0.51** (109:214)	<0.003	0.06 - 0.23
her^{1}/her^{2}	**<0.01** (3:476)	**0.39** (128:326)	<0.01	0.14 - 0.50
Controls				
$her^{1}/her^{l(2)mat}$; Dp(her^{+})/+	1.11 (411:370)	0.94 (559:594)	0.98	0.93 - 1.01
her^{1}/her^{2}; Dp(her^{+})/+	1.01 (268:266)	0.76 (287:376)	1.11	0.91 - 0.93

The her^{-} mothers (or control mothers) were crossed to fathers of the designated genotypes; all daughters inherit one (X-linked) *Sxl* or Sxl^{+} allele from the father. Sons are all Sxl^{+}. All crosses, 25°C.

*Data in III are from Pultz et al. (1994), included here for comparison to genotypes I and II. For IV, a range of data have been compiled from several Sxl^{+} experiments (Pultz et al., 1994 and M. A. Pultz, unpublished data).

Full genotypes: Sxl^{f9} = Sxl^{f9} ct^{6} *sn*. Sxl^{fLS} = *y* Sxl^{fLS} *oc v* f^{36a}. $Sxl^{f\#1}$ = $Sxl^{f\#1}$ *oc ptg v*. Dp (her^{+}) = Dp(2;3)*osp*^{3}, *Ki* her^{+}. her^{1}/her^{2} = her^{1} *or*/*SM1*, her^{2}, *Cy*. $her^{1}/herl^{(2)mat}$ = her^{1} *or*/*b* $herl^{(2)mat}$ (for Sxl^{f9} crosses, and for crosses with $Sxl^{f\#1}$ and Sxl^{+} from Pultz et al., 1994) or her^{1} *pr*/*b* $herl^{(2)mat}$ (for Sxl^{fLS} crosses) or her^{1} *pr*/$herl^{(2)mat}$ (for Sxl^{+} results from Pultz et al., 1994).

regulators of *Sxl*. Our previous work also showed that defective maternal *her* function makes daughters unable to tolerate a single copy of the null $Sxl^{f\#1}$ allele (Pultz et al., 1994), further suggesting that the maternal effect of *her* on female progeny is through an effect on zygotic *Sxl* expression. This view predicts that the daughters of *her* mutant mothers should be rescued if *Sxl* is expressed constitutively. We tested this prediction using the $Sxl^{M\#1}$ constitutive allele (Cline, 1978, 1979). Table 2A shows that $Sxl^{M\#1}$ restores female viability. For control her^{+} mothers, the viability of $Sxl^{M\#1}/Sxl^{+}$ daughters is 104% (relative to Sxl^{+} sons), and the relative viability of Sxl^{+}/Sxl^{+} daughters is 89%. For her^{-} mothers, the relative viability of $Sxl^{M\#1}/Sxl^{+}$ daughters is greater than 100%, whereas the viability of their Sxl^{+}/Sxl^{+} sisters is about 45% (relative to Sxl^{+} sons). Stated differently, for control mothers there is little difference in survival between $Sxl^{M\#1}$-bearing daughters and Sxl^{+} daughters; but for *her* mutant mothers, the $Sxl^{M\#1}$-bearing daughters are spared the morbidity of their Sxl^{+} sisters. These results suggest that the maternal effects of *her* on female viability are mediated through effects on *Sxl* expression.

$Sxl^{M\#1}$-bearing sons do not survive in the above experiment, regardless of maternal genotype. Thus, in males as in females, $Sxl^{M\#1}$ appears to function constitutively in a manner that is unaffected by the presence or absence of maternal *her* function.

To delimit further the relationship between maternal *her* function and *Sxl*, we examined interactions between *her* mutations and two function-specific *Sxl* alleles. Based on genetic criteria, the Sxl^{f9} allele is defective in the initiation of *Sxl* function, while the Sxl^{fLS} allele is defective in the maintenance of *Sxl* function (Cline, 1980, 1984; Sánchez and Nöthiger, 1982; Maine et al., 1985; Salz et al., 1987; Bernstein and Cline, 1994). The interaction between *her* and Sxl^{f9} is comparable to the interaction between *her* and a null allele for the *Sxl* locus**:** the survival of daughters is reduced to less than 10% of what is seen for Sxl^{+}/Sxl^{+} daughters (Table 2B). In contrast, *her* does not appear to interact significantly with the Sxl^{fLS} allele: the proportion of surviving daughters is no less than that obtained when *her* mothers are crossed to wild-type fathers. These results suggest that maternal *her* function is required for the initiation phase of *Sxl* expression. The results of all of the above experiments (with Sxl^{f9}, Sxl^{fLS} and $Sxl^{M\#1}$) are consistent with the model that maternal *her* function is important for female viability because it is needed for the initiation of *Sxl* function.

Maternal effects on *Sxl* expression

To examine whether maternal *her* function is needed as a positive regulator of the early female-specific *Sxl* promoter, we used the *lacZ* reporter gene construct of Keyes et al. (1992). Fathers homozygous for the reporter gene construct were crossed to her^{-} mothers and to her^{+} control mothers, and an anti-β-galactosidase monoclonal antibody was used to visualize protein expression. Among the progeny of control mothers, there was a striking difference between a class of darkly staining embryos and a class of lightly staining embryos (Fig. 1A). Each of these classes of embryos accounted for almost one-half of the progeny, although a few embryos showed variable intermediate staining levels. We assume that the darkest class of control embryos were females and the lightest were males. In contrast, none of the progeny of her^{-} mothers stained as intensely as the darkest class of control embryos; the staining levels were consistently more uniform among all progeny (Fig. 1B), as would be expected if daughters of her^{-} mothers are unable to fully activate the early female-specific *Sxl* promoter. Siblings of these embryos were assayed for viability; daughters of her^{-} mothers survived to adulthood about 4% as often as their brothers, whereas the daughters of control mothers survived as well as their brothers. This experiment suggests a role for maternal *her* products in the transcriptional activation of *Sxl*.

We also examined the expression of *Sxl* protein, using an anti-*Sxl* protein monoclonal antibody (Bopp et al., 1991). In this case, a weaker *her* allelic combination was used; about one quarter of the females survived to adulthood in this experiment. When progeny from her^{+} and her^{-} mothers were examined for

SXL expression, in each case approximately 50% of the embryos were stained (at least partially) and 50% were unstained, and we assume that the stained embryos represent the female progeny and, on this basis, we refer to them below as female (for quantitation, see Methods). Fig. 2A shows the uniform pattern of SXL expression typically seen in daughters of control *her*$^{+}$ mothers (of the stained control embryos, approximately 90% stained uniformly while about 10% lacked staining in a limited subset of cells). The prevailing staining pattern for these daughters appears identical to that reported for wild-type females by Bopp et al. (1991). In contrast, all daughters of *her*$^{-}$ mothers showed a patchy, uneven distribution of SXL staining: Fig. 2B shows a typical embryo, Fig. 2C,D illustrates the range of expression patterns observed. SXL expression was eliminated most often and most extensively in the ectoderm, with the affected segmental domain varying from embryo to embryo. Most embryos had several segments lacking SXL expression – often in the head, and most often in the mid-abdominal and/or thoracic segments. Generally, strong SXL expression was maintained in the extreme posterior of the abdomen, with weaker expression in the anterior and posterior midgut; in addition, expression tended to persist in cells of the mesodermal layer when it had been lost from other tissues. The observed effects on SXL expression directly support the hypothesis that maternal *her*$^{+}$ function is required for positive regulation of *Sxl*.

Maternal effects on *mle* protein expression

The maternal role of *her* in female development can be further defined by examining *her*'s effect on the regulation of dosage compensation. The above experiments indicate that maternal *her* products positively regulate *Sxl*, which functions in females to prevent hyper-transcription of the X chromosome (i.e., to prevent dosage compensation). When *Sxl* initiation is perturbed in females, some cells fail to turn off dosage compensation appropriately, resulting in a mosaic mixture of cells with female-like and male-like states of dosage compensation (Cline, 1984; Gorman et al., 1993). To ask whether daughters of *her* mothers show abnormalities in dosage compensation consistent with a defect in *Sxl* initiation, we examined binding patterns for the *mle* protein. In wild-type *Drosophila*, MLE is produced in both sexes; however, it binds along the X chromosome in males only (Kuroda et al., 1991).

In salivary gland polytene chromosomes from the daughters of control *her*$^{+}$ females, MLE did not bind preferentially to the

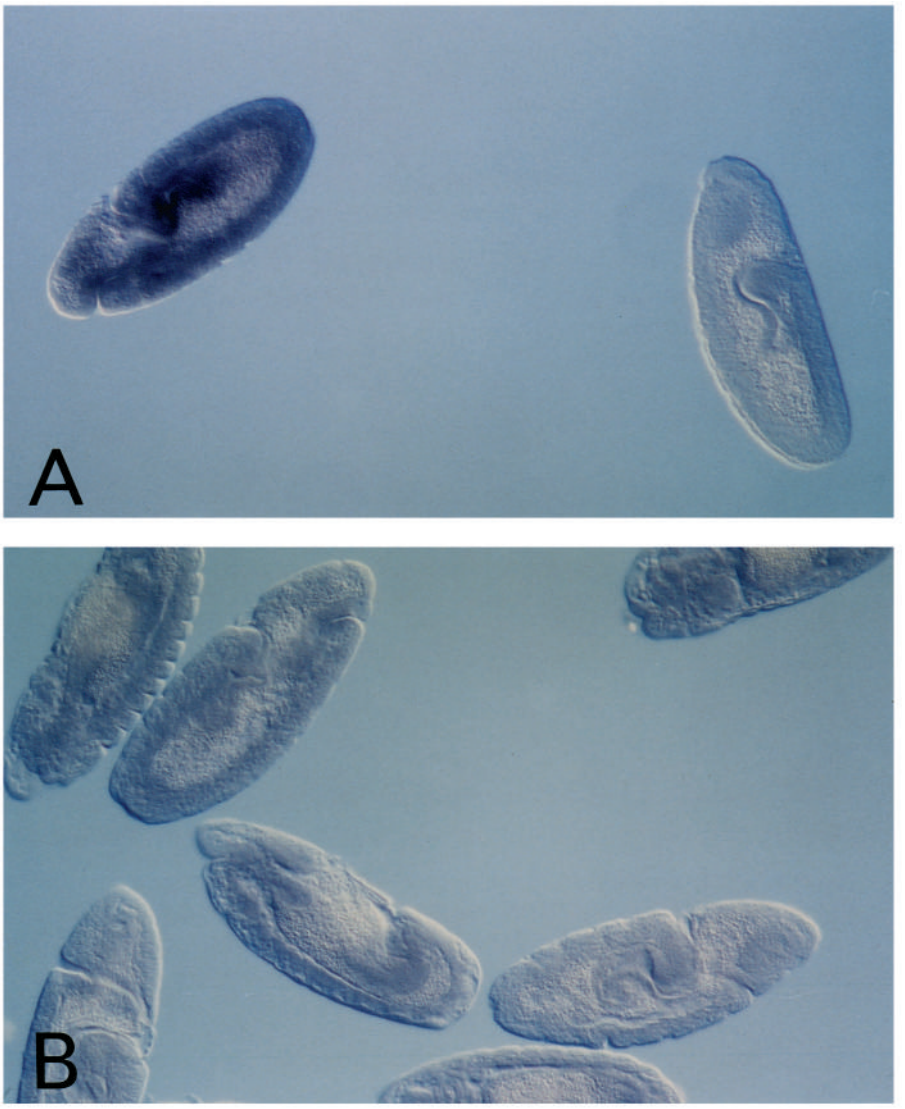


Fig. 1. Maternal *her* function and the expression of the *Sxl* early promoter. All embryos have a single copy of the lacZ reporter gene for the *Sxl* early promoter (Keyes et al., 1992), and the gene products are visualized using a monoclonal antibody against beta-galactosidase. (A) Progeny of control *her*$^{+}$ mothers – stained embryos are assumed to be female and unstained embryos are assumed to be male. (B) Progeny of *her* mothers – when treated identically to controls, no darkly staining embryos are observed (and the difference between the darkest and lightest embryos is always less than for controls), indicating defective *Sxl* transcription in female embryos.

Fig. 3. Maternal *her* function and the expression pattern of *mle* protein. Salivary chromosome preparations showing two cells from the same individual, a daughter of a *her*$^{-}$ mother (for full genotypes and crosses, see Methods). Chromosomes were simultaneously stained with anti-MLE primary antibodies followed by rhodamine-conjugated secondary antibodies (left) and with the DNA-binding stain Hoechst 33258 (right). The cell in the top panel shows weak background staining of all chromosomes, with no preferential staining of the X chromosome (on the far right), as is typical for female cells. The cell in the bottom panel shows preferential staining of the X chromosome (center), as is typical for male cells. Most daughters of *her*$^{-}$ mothers showed a mosaic mixture of cells with female-like and male-like MLE-binding patterns.

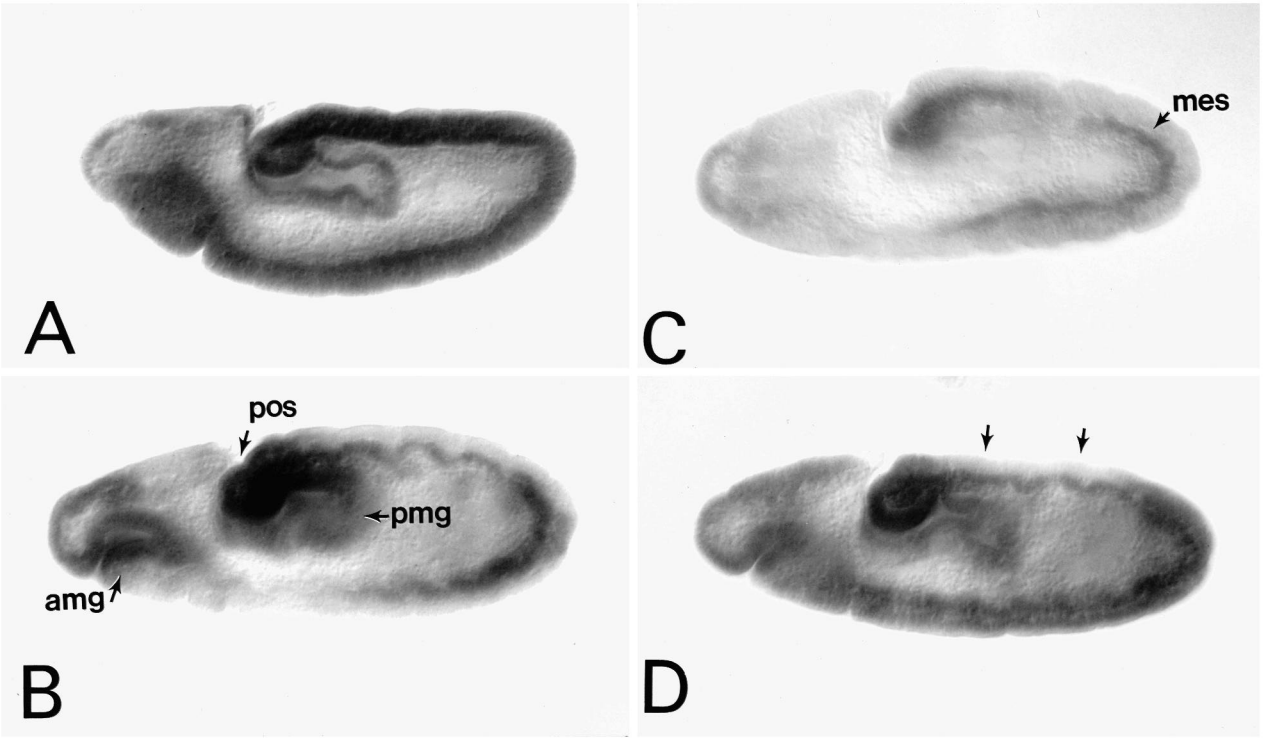
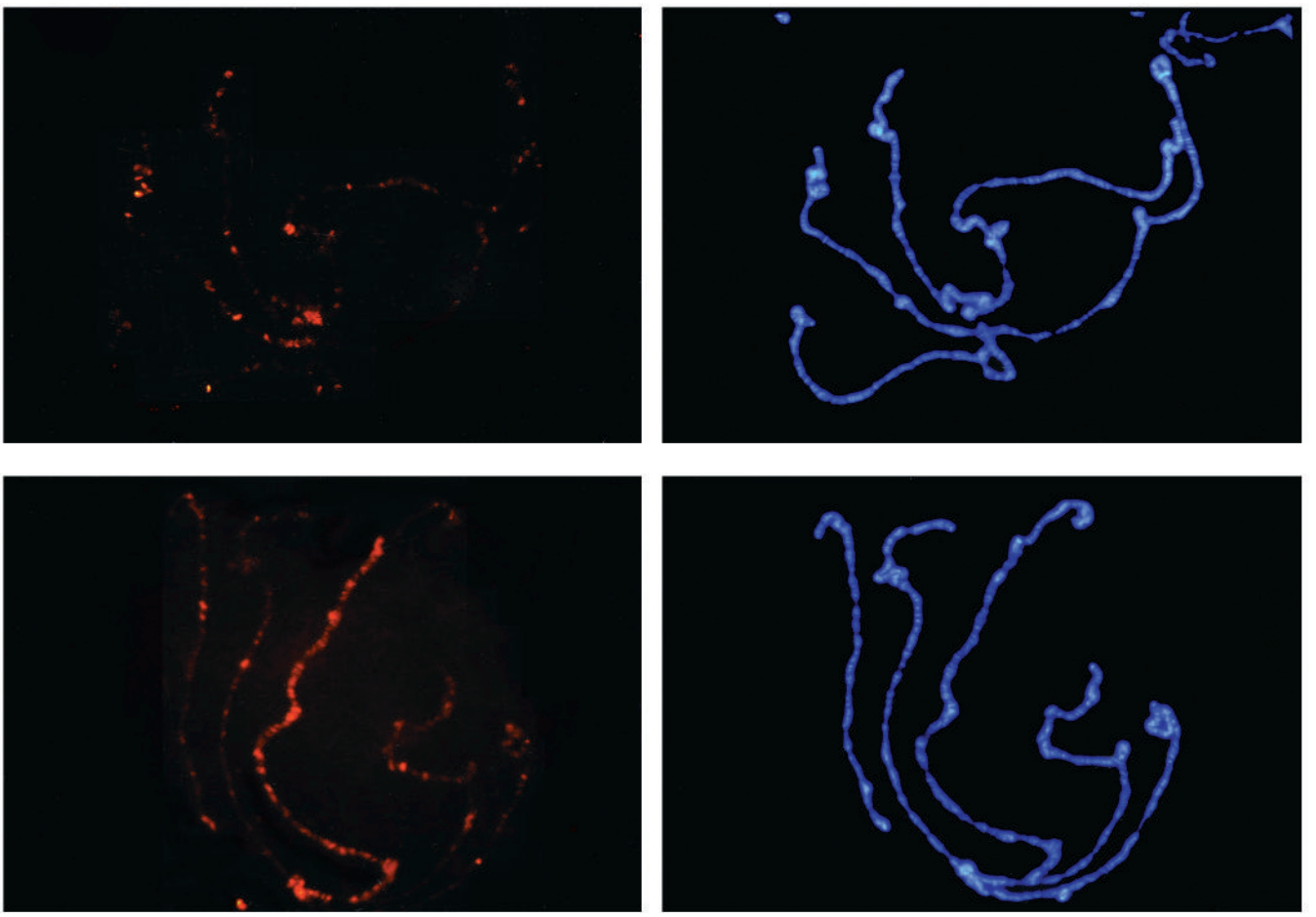


Fig. 2. Maternal *her* function and the expression pattern of *Sxl* protein. (A) Uniform SXL expression typically seen in daughters of her^{+} control mothers, (B-D) Nonuniform SXL expression seen in daughters of her^{-} mothers; amg: anterior midgut, pmg: posterior midgut, pos: posterior abdominal staining, mes: mesoderm; arrows in (D) indicate a patch of abdominal tissue in which SXL expression is lacking.



X chromosome (in 20/20 individuals) in agreement with previous results for wild-type females (Kuroda et al., 1991). In contrast, in the salivary glands of the daughters of *her*$^{-}$ mothers, most glands (22/25) were mosaic with regard to their MLE staining pattern (Fig. 3): some cells exhibited a normal female-like MLE-binding pattern, with no preferential binding to the X chromosome, while other cells exhibited a male-like pattern, with extensive binding of MLE along the X chromosomes. The remaining daughters, 3/25, expressed MLE in a female-like binding pattern in all cells. In some salivary glands with mosaic patterns of MLE staining, cells were observed with a male-like MLE-binding pattern on both of the unpaired X chromosomes (not shown), ensuring that the male-like cells are indeed diplo-X, rather than having been sexually transformed through X-chromosome loss. Typically, within a given mosaic individual, the male-like MLE-binding pattern was seen in several cells located in close proximity to one another, suggesting that this male-like condition was shared by a group of cells with a common clonal origin. In any given individual, the population of cells expressing the male-like MLE-binding pattern tended to have smaller (presumably underreplicated) polytene chromosomes than the cells expressing the female-like pattern. Some, but not all of the diplo-X cells expressing MLE in a male-like pattern showed a diffuse X-chromosome morphology, typical of male X chromosomes. The salivary gland chromosomes in daughters of *her*$^{-}$ mothers appeared to be either distinctly female-like or distinctly male-like in their MLE-binding pattern; we could detect no evidence of a gradation of MLE-binding patterns intermediate between male-like and female-like states. Thus it appears that lack of maternal *her* function results in a mosaic pattern of cells expressing dosage compensation functions in either female-like or male-like patterns in those daughters that have survived to the third larval instar stage. These results are consistent with a role for maternal *her* products in regulating the initiation of *Sxl*.

Taken together, all of the the above experiments indicate that maternal *her* function is necessary for the normal positive regulation of *Sxl* in female offspring, and that the effect of *her* on *Sxl* accounts for the female-specific maternal effects of *her* on viability. For a summary of these experiments and a model for maternal *her* function in the sex determination regulatory hierarchy, see Fig. 4.

Zygotic effects of *her* on female sexual differentiation

Having asked where *her* functions maternally in the sex determination regulatory hierarchy to ensure the viability of female progeny, we now ask where *her* functions zygotically in that hierarchy to ensure proper female sexual differentiation. Can the *her* female sexual differentiation phenotype be rescued by constitutive *Sxl* or or constitutive *tra* expression? Does defective zygotic *her* function affect the sex-specific splicing of *dsx* transcripts?

Zygotic *her* function and *Sxl*

Since the maternal effect of *her* on female viability is due to its role as a positive regulator of *Sxl*, it seemed plausible that the zygotic effects of *her* on female sexual differentiation might also be through regulation of *Sxl*. Therefore, we tested whether the constitutive allele *Sxl*$^{M\#1}$ can rescue the intersexual phenotype of *her* females. Table 3A shows that *her*$^{-}$ females bearing the *Sxl*$^{M\#1}$ chromosome have an intersexual phenotype indistinguishable from that of their *Sxl*$^{+}$*;her*$^{-}$ sisters. Since constitutive *Sxl* expression cannot ameliorate the intersexuality of diplo-X *her*$^{-}$ individuals, the zygotic aspect of *her* function must not be acting through *Sxl* – at least not by controlling any aspect of *Sxl* expression that occurs constitutively in the *Sxl*$^{M\#1}$ allele. This suggests that zygotic *her* function differs from maternal *her* function in the level at which it is required within the sex determination regulatory hierarchy.

Zygotic *her* function and *tra*

We then asked whether the zygotic function of *her* is acting at the next step of the sex determination hierarchy, by regulating *tra*. Normally, *Sxl* controls *tra* function by directing a female-specific splice choice that must be taken in order to generate a protein-coding *tra* mRNA (reviewed in Baker, 1989; Steinmann-Zwicky et al, 1990; Belote, 1992; Mattox et al., 1992; McKeown and Madigan, 1992). To ask whether zygotic

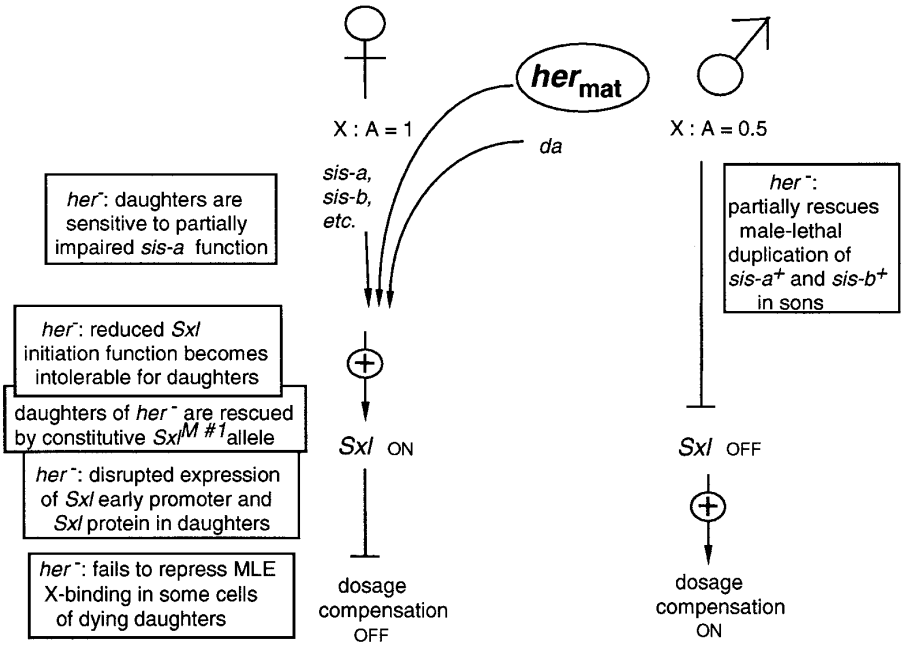


Fig. 4. Model of maternal *her* function and summary of experiments. The proposed maternal role of *her* in the sex determination hierarchy is shown, with a summary of the experimental evidence for this role. Shown in the figure are regulatory genes addressed in the experiments (referenced in text), as well as *da*, the first characterized maternal regulator of *Sxl* (Cline, 1976, 1980, 1993; Cronmiller and Cline 1987). Activity of *Sxl* is controlled by additional positive and negative regulators not shown here. Positive regulators include *runt, sans-fille (snf)* and *fl(2)d* (Cline, 1988; Oliver et al., 1988; Steinmann-Zwicky, 1988; Duffy and Gergen, 1991; Granadino et al., 1992; Salz, 1992; Albrecht and Salz, 1993; reviewed in Cline, 1993). Negative regulators include *hairy*, *deadpan* and *extramacrochaete* (Parkhurst et al, 1990; Younger-Shepherd et al., 1992).

Table 3. Zygotic effects of *her*

(A) Can the *her* intersexual phenotype be rescued by constitutive *Sxl* expression?

	Genotypes			
	her^{1}/+		*her*^{1}	
	with *Sxl*$^{M\#1}$	without *Sxl*$^{M\#1}$	with *Sxl*$^{M\#1}$	without *Sxl*$^{M\#1}$
XX individuals				
female	465	440	0	0
intersex	0	0	161	126
XY individuals				
male	0	463	0	147*

Cross: *Sxl*$^{M\#1}$,*cm*$^{+}$/*cm*; *her*^{1}/ *SM1* females × *cm*; *her*^{1}/ *SM1* males, 25°

Sxl and *cm* are extremely closely linked, (0 recombinants in >10,000 scored – Cline, 1978), therefore *Sxl*$^{M\#1}$ can be scored as *cm*$^{+}$.

**her* males have normal male pigmentation and genitalia; presence of a few sixth sternite bristles may indicate slight intersexuality - see also Discussion and Pultz et al. (1994).

Full genotypes: *her*^{1} = *b her*^{1} ; *Sxl*$^{M\#1}$ = *y w* ^{a}*Sxl*$^{M\#1}$ *sn*. The SM1 balancer chromosome is *her*$^{+}$ and is marked with *Cy*. For further comment, see Methods.

(B) Can the *her* intersexual phenotype be rescued by constitutive *tra* expression?

	Genotypes					
	hs-tra/balancer				TM3/TM6B*	
	XX; *her*^{1}/+	XY; *her*^{1}/+	XX; *her*^{1}	XY; *her*^{1}	XX; *her*^{1}/+	XY; *her*^{1}/+
phenotypically female	470	380	0	0	111	0
phenotypically intersexual	0	2	12	8	0	0
phenotypically male	0	0	0	0	0	86

Cross: *her*^{1}/*SM1*; *hs-tra/TM6B* × *B*^{s}*Y; her*^{1}*/SM1; TM3/TM6B*, 25°C.

"hs-tra" refers to the construct of McKeown et al. (1988).

*No individuals of the *her*^{1}/*her*^{1};*TM3/TM6B* class survived at 25°C. This is probably due to combined negative effects of her plus two third chromosome balancers on viability. That her flies tolerate balancer chromosomes poorly probably also accounts for the low number of her individuals surviving in the hs-tra/balancer class.

Full genotypes: *her*^{1} *or/SM1,her*$^{+}$ *Cy; hs-tra, Dfst*^{J7} *Ki pP/TM6B×B*^{s}*Y; her*^{1} *or/SM1, her*$^{+}$ *Cy; TM3,Sb pP/TM6B*. The *hs-tra* bearing individuals were identified by the *Ki* marker, *B*^{s} individuals were assumed to be XY (*B*$^{+}$ to be XX). The *her*^{1} individuals were identified as *Cy*$^{+}$ individuals, small and with roughened eyes - the latter phenotypes are sometimes associated with the reduced viability of *her* individuals of both sexes (Pultz et al. 1994), and were especially pronounced in this genetic background. One *Cy*$^{+}$ individual (XX, *hs-tra*/TM3) was normal in size, eye morphology and female sexual development. We believe that this individual was probably not a *her*^{1} individual but rather a rare case in which *Cy* had been lost by recombination from the balancer chromosome. Reciprocal events (*her Cy* chromosomes) have been recovered in this stock at similar frequencies (not shown).

her function might also regulate *tra*, we determined whether the *her* female sexual differentiation phenotype can be rescued by expression of *tra* from the molecularly constructed constitutive allele of McKeown et al. (1988). This construct overrides the normal control of *tra* both at the level of transcription and at the level of sex-specific splicing: a *tra* cDNA clone, spliced in the female mode to encode a functional polypeptide, is placed under transcriptional control of the *hsp70* promoter. Even under non heat-shock conditions, basal levels of transcription from this promoter are sufficient to feminize XY individuals such that they are transformed to females in their external morphology. Table 3B shows that under conditions where constitutive expression of *tra* is sufficient to transform *her*$^{+}$ XY individuals to female morphology, *her*$^{-}$ diplo-X individuals bearing the *hs-tra* construct are still intersexual. In addition, *her*$^{-}$ XY individuals bearing *hs-tra* are intersexual (equivalent in external morphology to XX *her*$^{-}$ individuals), presumably having been fully feminized by the *hs-tra* construct but transformed to intersexes due to the lack of *her* function. Therefore, zygotic *her* function does not appear to influence female sexual differentiation by regulating the transcription or female-specific splicing of *tra*.

Zygotic *her* function and *dsx*

Next, we asked whether *her* regulates the next step of the pathway, the female-specific expression of *dsx*. Normally, *dsx* is transcribed in both sexes and sex-specific regulation takes place at the level of splicing. When *tra* protein is present a female-specific *dsx* mRNA is generated, encoding a female-specific version of the *dsx* protein. In the absence of *tra* function, a male-specific mRNA is generated by default, encoding a male-specific version of the *dsx* protein (Nagoshi et al., 1988; Burtis and Baker, 1989).

In the absence of all products of the *dsx* locus, diplo-X individuals are transformed to intersexes; thus the intersexual phenotype of *her*$^{-}$ individuals could be due to a failure to express any *dsx* gene products. However, there is also another (seemingly opposite) mechanism by which diplo-X individuals can be transformed to intersexes through *dsx* misfunction: an approximately even mixture of female-specific *and* male-specific *dsx* transcripts can also result in intersexuality, presumably because the female-specific and male-specific *dsx* proteins interfere with one another's ability to function (Baker and Ridge, 1980; Nöthiger et al., 1987; Nagoshi and Baker, 1990). Thus the intersexual *her* phenotype could also result from a partial misregulation of *dsx* splicing, generating a mixture of female-specific and male-specific *dsx* products.

We explored both of the above possibilities through northern blot analysis, asking whether *dsx* transcripts are affected in diplo-X *her*$^{-}$ intersexes. These results are shown in Fig. 5: *dsx* transcripts are present in diplo-X *her*$^{-}$ intersexes and are indistinguishable in size from the female-specific *dsx* transcripts seen in *her*$^{+}$ females. No transcripts are seen in the size range expected for male-specific *dsx* products. Thus it appears unlikely that *her* affects female sexual development by impairing the expression or female-specific splicing of *dsx* transcripts.

Taken together, the above experiments indicate that *her* functions zygotically at the last step of the known sex determination hierarchy. For a summary of these experiments and model for the zygotic role of *her* in the sex determination hierarchy, see Fig. 6.

DISCUSSION

We have examined the maternal and zygotic roles of *hermaphrodite* (*her*) in female-specific development, and find that *her* function is required at two distinctly different levels of the sex determination regulatory hierarchy. Maternal *her* function is required early in the hierarchy for the positive regulation of *Sxl* (Fig. 4); whereas zygotic *her* function is required late in the hierarchy for female sexual differentiation (Fig. 6).

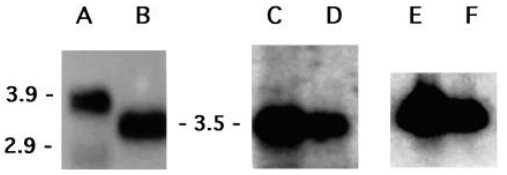


Fig. 5. Zygotic *her* function and *dsx* expression. Total RNA was prepared from wild-type males (A) and females (B), her^1/+ control females (C,E) and her^1/her^1 intersexual females (D,F). Lanes A-D were probed with a single-stranded DNA probe which recognizes both the female-specific and male-specific *dsx* mRNAs by the method of Burtis and Baker (1989). The blot containing lanes C and D was reprobed with a probe recognizing ribosomal protein 49 (see Methods) to assay the relative amounts of RNA in each lane. Adult flies were used for these experiments, as previous work has shown that other genes in the hierarchy such as *Sxl*, *tra* and *tra-2* regulate *dsx* continuously during adult life (Nagoshi et al., 1988).

Maternal *her* function appears to affect the same process as *sis-a*, *sis-b* and an initiation-defective *Sxl* allele; in addition, daughters can be rescued from a lack of maternal *her* function by constitutive *Sxl* expression. In molecular terms, inadequate maternal *her* function fails to activate the early *Sxl* promoter fully, leaving daughters with reduced and patchy embryonic *Sxl* protein expression. Dosage compensation activity is also misregulated, as indicated by an inappropriate male-like MLE-binding pattern in a subset of salivary gland cells from dying daughters. Thus maternal *her* function is necessary as a positive regulator of *Sxl*, and the lethality of daughters is most likely due to dosage compensation upsets that ensue with insufficient *Sxl* expression.

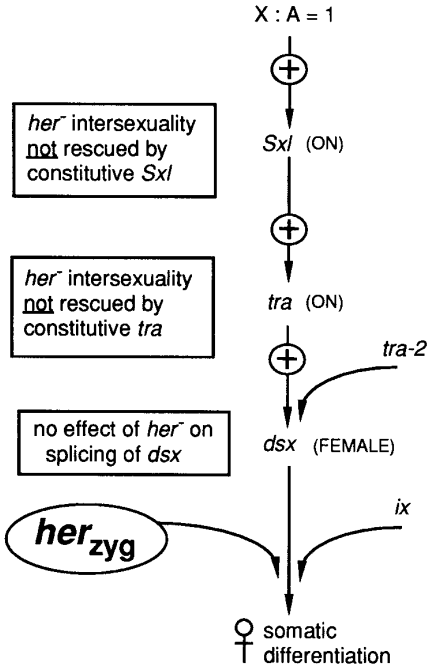


Fig. 6. Model of zygotic *her* function and summary of experiments. The proposed zygotic role of *her* in female sexual differentiation is shown relative to the sex determination regulatory hierarchy, with a summary of experimental results.

The zygotic effects of *her* on female sexual differentiation cannot be rescued by constitutive *Sxl* expression, nor can they be rescued by constitutive expression of *tra*. Moreover, a lack of *her* zygotic function transforms females to intersexes without obviously impairing the expression or splicing of *dsx* transcripts. Thus *her*'s zygotic role is distinct from its maternal role in the sex determination hierarchy. The zygotic role of *her* is also distinct from the roles of *Sxl*, *tra* and *tra-2*, which are all required (directly or indirectly) for the female-specific control of *dsx* splicing. Rather, the female-specific zygotic function of *her* – like that of the formally comparable *ix* gene (Baker and Ridge, 1980, Nagoshi et al., 1988) – is required in parallel to, or downstream of, *dsx* for normal female sexual differentiation.

The maternal role of *her* is similar in several respects to the maternal role of *daughterless (da)* in the regulation of *Sxl* function (Cline, 1976, 1978, 1988, 1993; Maine et al., 1985). For example, a lack of either maternal *her* function or maternal *da* function can rescue males from an otherwise deleterious duplication of *sis-a* and *sis-b*, suggesting that maternal contributions from both genes are required to enable the *sis* genes to turn on *Sxl* effectively. Both *her* and *da* interact with the 'initiation-specific' allele Sxl^{f9} and not with the 'maintenance-specific' allele Sxl^{fLS}. Furthermore, daughters of both her^- and da^- mothers can be rescued by constitutive *Sxl* expression from $Sxl^{M\#1}$. The converse is not true: loss of maternal *her* function does *not* rescue sons from the male-lethal effects of the constitutive $Sxl^{M\#1}$ allele. Similarly, loss of maternal *da* function does not rescue $Sxl^{M\#1}$ male-lethal effects; although this male lethality is partially suppressed by loss of *sans-fille* (*snf*)or *fl(2)d* function (Cline, 1978,1986; Steinmann-Zwicky, 1988; Granadino et al., 1992). The similarities between *her* and *da* suggest that maternal *her* function is needed for the initiation of *Sxl* expression.

To test the maternal role of *her* in regulating *Sxl*, we examined the expression of a reporter gene driven by the early *Sxl* promoter (Keyes et al., 1992). Loss of maternal *her* function reduces the expression of the reporter gene relative to controls, consistent with a maternal role for *her* in the transcriptional initiation of *Sxl* expression. We also examined the pattern of *Sxl* protein expression in embryos. When maternal *her* function is defective, SXL is expressed in variable non-uniform patterns. However, some tissues of the embryo are more resistant than others to a loss of SXL expression, including the anterior and posterior midgut, the extreme posterior of the abdomen, and the mesodermal cell layer. The observed persistence of *Sxl* in the mesoderm is interesting in light of the reported collaboration of *dorsal (dl)*, *da* and other helix-loop-helix proteins in the control of mesodermal gene expression (González-Crespo and Levine, 1993); perhaps some of these other genes are contributing to the control of *Sxl* expression in mesodermal cells. The persistent patterns of SXL expression in daughters of *her* mutant mothers overlap partially with the SXL patterns associated with partial loss of *da* or *sis-b* expression. In particular, persistent SXL expression in the anterior and posterior midgut appears to be shared in embryos that are lacking in maternal *her*, maternal *da* or zygotic *sis-b* expression (Bopp et al., 1991). However, daughters of da^1 mothers also have a persistent SXL expression in the posterior head segments that is not observed in daughters of her^- mothers. Loss of SXL expression in daughters of her^- mothers often extends anteriorly beyond the central domain of the

embryo where *runt* is required for SXL expression (Duffy and Gergen, 1991). Taken together, these observations indicate that *her* is one of several genes with additive yet distinctive contributions to the control of *Sxl* expression in various regions and tissues of the embryo.

The role of maternal *her* product in regulating *Sxl* is reflected in an effect on the regulation of dosage compensation. When maternal *her* function is defective, those daughters that have made it to the end of the larval period are clearly embarked on a self-destructive course, having failed to turn off the X-chromosome hypertranscription machinery properly. These moribund daughters are mosaics of cells that have settled into either female-like or male-like dosage compensation states, as assayed by the binding of *mle* protein to X chromosomes in salivary glands. Such mosaics are also observed in the salivary glands of females with partially impaired *Sxl* function due to the $Sxl^{fhv\#1}$ mutation (Gorman et al., 1993). With a loss of maternal *her* function, diplo-X cells with a male-like MLE-binding pattern have polytene chromosomes that appear on average to be smaller than their counterparts with the appropriate female MLE pattern. The apparent poor health of those cells may be caused by overexpression of the X chromosome (due to the inappropriate MLE binding); however, individuals that are similarly mosaic for MLE binding due to a mutant *Sxl* genotype (diplo-X $Sxl^{fhv\#1}/Sxl^{null}$) do not display any notable differences in polytene chromosome size (M. Gorman, personal communication). Perhaps a lack of maternal *her* function prevents *Sxl* activity sufficiently early and/or sufficiently completely to be reflected in apparent difficulties in cell growth by the third instar larval stage, whereas effects on cell viability in $Sxl^{fhv\#1}/Sxl^{null}$ females are developmentally delayed (because of later or incomplete loss of *Sxl* activity). It is also possible that, in the progeny of *her* mothers, the difficulties of 'male-like' cells are exacerbated by a partial loss of additional (sex non-specific) maternal *her* function(s) (Pultz et al., 1994).

When maternal *her* function is lacking, we assume that those larval cells with a male-like state of dosage compensation have also misassessed their state of sexual differentiation (as 'male-like'). However, since surviving adult her^+ daughters of her^- mothers are not sexually mosaic, we assume that those cells that have misassessed both dosage compensation and sexual identity as 'male-like' do not survive to produce external sexually dimorphic structures in the adult. Consistent with this interpretation is the finding that if dosage-compensation gene function is eliminated in these mosaic daughters of her^- mothers, thereby reestablishing the female X chromosome transcription rate, cells with male-like morphology now survive and contribute to mosaic intersexual structures (G. Carson and M. A. Pultz, unpublished results). These results are similar to the results obtained when dosage compensation gene function is removed from *Sxl* individuals (Skripsky and Lucchesi, 1982; Uenoyama et al., 1982; Cline, 1984; Gorman et al., 1993) or from daughters of da^1 mothers (Cline, 1982, 1984). Thus the observed relationship between maternal *her* function and dosage compensation is concordant with current understanding of the regulatory hierarchy governing dosage compensation and sexual differentiation.

In contrast to *her* maternal function, *her* zygotic function is required at the last known step of the sex determination regulatory hierarchy. There is no indication that *her* affects the expression or female-specific splicing of *dsx* transcripts. In regulating female sexual differentiation, the function of *her* may be similar to the function proposed for *ix* (Burtis and Baker, 1989) – collaborating with *dsx* to control female-specific gene expression. This view of *her* function also accommodates the observation that *her*, unlike *ix*, also has a possible role in male sexual differentiation. Partial loss of zygotic *her* function results not only in a strong intersexual transformation of females, but also in what is probably a weak intersexual transformation of males (Pultz et al., 1994); whether a complete loss of *her* function would result in a strong intersexual transformation of males is not yet known. Interestingly, partial loss of *dsx* function also has strong effects on female sexual development while only weakly affecting males, according to descriptions of six hypomorphic *dsx* alleles (Nöthiger et al., 1987; Baker et al., 1991). Thus a partial loss of either *her* or *dsx* function impacts sexual differentiation more strongly in females than in males. The greater sensitivity of females to partial loss of these zygotic regulatory gene functions might reflect greater complexity in the control of female sexual differentiation. For example, in contrast to male development, female sexual differentiation also requires participation of the *ix* gene (Baker and Ridge, 1980). Alternatively, the shorter carboxyl terminus of the female-specific *dsx* protein, with a female-specific sequence of 30 amino acids, might make it less robust in function or stability than the male-specific *dsx* protein, with a male-specific sequence of 152 amino acids (Burtis and Baker, 1989). In any case, the similar phenotypes associated with partial loss of *her* or *dsx* function suggest that these genes may act together to regulate sexual differentiation in both sexes.

This view is also consistent with preliminary evidence from experiments examining *her* mutant phenotypes in the presence of the constitutively expressed male-specific dsx^M allele. Transcripts of *dsx* are normally expressed from a sex non-specific promoter, and regulated sex-specifically at the level of splicing. The dsx^M allele cannot be spliced in the female-specific mode, so its transcripts are spliced to the male-specific form in both sexes (Nagoshi and Baker, 1990). With only the male-specific functional gene products, dsx^M/dsx^{null} XX individuals develop as normal males with respect to their external morphology (Baker and Ridge, 1980). However, although dsx^M can masculinize XX individuals, it does not affect the weakly intersexual phenotypes of her^- males (Pultz, unpublished data). It is clear that the dsx^M allele can be expressed in the absence of *her* function. In chromosomal females, such expression is manifested in the phenotype of $her^-;dsx^M/dsx^{null}$ diplo-X individuals, which are externally masculinized relative to their $her^-;dsx^+/dsx^{null}$ intersexual sisters (dsx^M-bearing her^1 females appear similar to her^1; dsx^+ males). Together, these results suggest that for males as for females *dsx* gene products are normally expressed but not fully functional when zygotic *her* function is lacking.

The functions of *her* in the sex determination regulatory hierarchy suggest to us that *her* may encode a transcriptional regulatory protein. First, the maternal function of *her* is needed early in the hierarchy and appears to be necessary for expression of transcripts from the early *Sxl* promoter. The similarities between maternal *her* function and the sex-determining functions of *da* and *sis-b* are also consistent with this view. The latter encode helix-loop-helix proteins that bind DNA as

heterodimers and may directly initiate *Sxl* expression (reviewed in Cline, 1989, 1993). Second, the zygotic function of *her* is needed at the end of the sex determination regulatory hierarchy, where control of gene expression returns to the transcriptional level after a cascade of regulated splicing – the *dsx* gene encodes zinc-finger-related proteins that bind in vitro to the female-specific enhancer region of a yolk protein gene (Burtis et al., 1991; Coschigano and Wensink, 1993; Erdman and Burtis, 1993). Thus, *her* function is required at both ends of the regulatory hierarchy, at two different levels where transcriptional regulation is key.

Finally we note that the general theme described here for *her*, that a single gene can function at more than one level in the sex determination regulatory hierarchy, is related conceptually to the theme described for work comparing the regulatory relationships between *Sxl* and *dsx* in somatic versus germline sexual differentiation. Although *Sxl* clearly controls *dsx* expression in the female soma (Nagoshi et al., 1988), in contrast, a *dsx*-mediated signal must be received from the soma before *Sxl* can be secondarily expressed in female germline tissue (Oliver et al., 1993; Steinmann-Zwicky, 1994). Thus *Sxl* acts both 'upstream' and 'downstream' of *dsx* in the cascade of regulatory relationships in the soma and germline that lead to female germline differentiation. The results presented here explore the role of *her* only in somatic sexual differentiation and are thus not directly comparable to the *Sxl* studies. However, we find it intriguing that the regulatory cascade governing female sexual differentiation may be built on the principle of recapitulating regulatory gene function to a degree not previously suspected.

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