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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

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SUMMARY

The zygotic function of the *hermaphrodite* (*her*) gene of *Drosophila* plays an important role in sexual differentiation. Our molecular genetic characterization of *her* suggests that *her* is expressed sex non-specifically and independently of other known sex determination genes and that it acts together with the last genes in the sex determination hierarchy, *doublesex* and *intersex*, to control female sexual differentiation. Consistent with such a terminal function in sexual differentiation, *her* encodes a protein with C_2H_2-type zinc fingers. The *her* zinc fingers are atypical and similar to the even-numbered zinc fingers of ZFY and ZFX proteins in humans and other vertebrates.

Key words: *Drosophila*, *doublesex*, *hermaphrodite*, ZFY, Sexual differentiation

INTRODUCTION

Sex determination and differentiation in *Drosophila melanogaster* is controlled by a hierarchy of regulatory genes (Fig. 1; reviewed, for example, by Burtis, 1993; Burtis and Wolfner, 1992; Cline and Meyer, 1996; McKeown, 1992; Parkhurst and Meneely, 1994). The key element that determines whether a fly becomes a female or a male is the on/off state of the *Sex-lethal* (*Sxl*) gene (reviewed by Cline and Meyer, 1996; Sánchez et al., 1994). *Sxl* is on in females and off in males. During early stages of embryonic development, *Sxl* is sex-specifically controlled at the level of transcription by the relative levels of several transcription factors which act through *Sxl*'s early establishment promoter (P_e) (Fig. 1). Later in development, *Sxl* activity is maintained in females through autoregulation at the level of splicing. In females, *Sxl* controls regulatory genes that govern somatic sex determination and dosage compensation (reviewed by Baker et al., 1994; Cline and Meyer, 1996) and is also involved in germline sex determination (reviewed by Mahowald and Wei, 1994). *Sxl* has no function in males, where no full-length SXL protein is produced.

With respect to its role in female somatic sexual differentiation, the SXL protein directs the splicing of the *transformer* (*tra*) pre-mRNA to generate a functional mRNA in females whereas, in males, *tra* pre-mRNA is spliced in a default pattern that leaves premature stop codons in the mRNA (reviewed by Burtis, 1993; McKeown, 1992). Downstream of *tra*, the somatic sex determination pathway splits into two branches: one contains the *doublesex* (*dsx*) gene (Fig. 1; Baker and Ridge, 1980) and the other the *fruitless* (*fru*) gene (Fig. 1; Ryner et al., 1996).

In females, the *tra* gene product acts together with the *transformer-2* (*tra-2*) gene products, which are sex non-specifically expressed in the soma (Amrein et al., 1988; Goralski et al., 1989; Mattox et al., 1990), to direct the splicing of the *dsx* pre-mRNA to generate a female-specific mRNA (reviewed by Burtis, 1993; McKeown and Madigan, 1992). In males, where functional *tra* product is absent, default splicing of *dsx* pre-mRNA produces the male-specific *dsx* mRNA. *dsx* encodes sex-specific transcription factors that are required for all aspects of somatic sexual differentiation outside of the CNS (Burtis et al., 1991). In addition, wild-type *dsx* function is required for some aspects of sexual differentiation that do, or may, involve the CNS (Taylor and Truman, 1992; Villella and Hall, 1996).

The female-specific DSX protein (DSX^F) acts together with the products of the *hermaphrodite* (*her*) (Pultz and Baker, 1995) and the *intersex* (*ix*) genes (Chase and Baker, 1995; Erdman et al., 1996) to repress male differentiation and to promote female differentiation in females; conversely, the male-specific DSX protein (DSX^M) acts to repress female differentiation and to promote male differentiation in males (Jursnich and Burtis, 1993; Taylor and Truman, 1992; reviewed by Burtis, 1993; McKeown and Madigan, 1992). The only known direct target genes of *dsx* are the *yolk protein* (*yp*) genes *yp1*, *yp2* and *yp3*, which are terminal differentiation genes. Transcription of the *yp* genes in fat body cells is directly activated by DSX^F in females and inhibited by DSX^M in males (reviewed by Bownes, 1994).

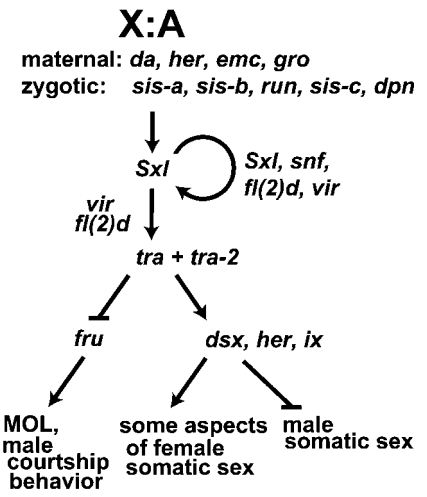

Fig. 1. *Drosophila* somatic sex determination hierarchy. For recent reviews of the hierarchy, see Parkhurst and Meneely (1994), and Cline and Meyer (1996). For additional information not included in the reviews regarding *female-lethal (2)d (fl(2)d)*, see Granadino et al. (1996). X:A represents the ratio of the number of X chromosomes to the number of sets of autosomes in a zygote and is the primary sex determination signal. *daughterless* (*da*), *hermaphrodite* (*her*), *sisterless-a* (*sis-a*), *sisterless-b* (*sis-b*), *sisterless-c* (*sis-c*), and *runt* (*run*) positively regulate the *Sxl* early promoter. *extra machrochaetae* (*emc*), *groucho* (*gro*), and *deadpan* (*dpn*) negatively regulate the *Sxl* early promoter. *virilizer* (*vir*), *sans fille* (*snf*) and *fl(2)d* are involved in the regulation of *Sxl*. See text for descriptions of other genes in the hierarchy. Arrows indicate positive regulation, bars indicate negative regulation.

The *tra* and *tra-2* products also direct the splicing of *fru* pre-mRNA into a female-specific mRNA (Ryner et al., 1996). In males, default splicing of *fru* pre-mRNA produces the male-specific *fru* mRNAs (Ryner et al., 1996). The male-specific *fru* products act only in a small part of the CNS where they are necessary for male sexual behavior (Hall, 1994; Ito et al., 1996; Ryner et al., 1996; Taylor et al., 1994) and the development of a male-specific abdominal muscle, the Muscle of Lawrence (MOL) (Gailey et al., 1991; Ito et al., 1996; Lawrence and Johnston, 1986; Ryner et al., 1996). The female-specific *fru* products have no known functions.

Most genes in the somatic sex determination pathway have been studied extensively both genetically and molecularly. The *her* gene is one of the few known genes in the pathway that has not been molecularly characterized. Genetic studies (Pultz and Baker, 1995; Pultz et al., 1994) have shown that *her* functions both maternally and zygotically. In addition, these studies have shown that maternally, as well as zygotically, *her* has certain functions that are involved in sex determination/differentiation and other functions that are essential for both sexes. Although the exact nature of the sex non-specific vital functions of *her* are unknown, significant insight has been gained into the nature of *her*'s sex determination/differentiation functions (Pultz and Baker, 1995; Pultz et al., 1994). The maternal sex determination function of *her* is required for the activation of the early promoter of *Sxl* (Pultz and Baker, 1995). It is unknown whether the *her* maternal sex-specific function regulates the *Sxl* early promoter directly, or indirectly, through other regulators of *Sxl* (reviewed by Cline and Meyer, 1996). With respect to the zygotic sex differentiation function of *her* in females, it was shown that *her* does not regulate the expression of *Sxl*, *tra* or *dsx* at the level of either transcription or the splicing of their pre-mRNAs (Pultz and Baker, 1995). These results led to the suggestion that the female-specific zygotic function of *her* acts in parallel with, or downstream of, *dsx* (Pultz and Baker, 1995). However, it was unknown whether *her* is sex-specifically regulated in zygotes and whether it is a downstream target of one or more of the somatic sex determination genes.

Whether the zygotic function of *her* is involved in sexual differentiation of males is unclear. While *her* mutant males appear slightly intersexual with respect to a very limited subset of sexual dimorphisms (for example, *her* mutant males have extra bristles on sternite 6), it is not known whether these effects are due to changes in sexual differentiation, or changes in other aspects of differentiation, such as segmental identity (Pultz and Baker, 1995; Pultz et al., 1994).

We report here a molecular genetic characterization of the *her* gene. *her* encodes a single protein with four C_2H_2-type zinc fingers. *her* transcripts are supplied maternally to embryos and are expressed zygotically in both sexes. The transcription and the splicing of *her* pre-mRNAs are independent of other known sex determination genes. We show that *her* functions in conjunction with the *dsx* branch, but not the *fru* branch, of the sex determination hierarchy. Our results suggest that *her* is expressed sex non-specifically and acts together with *dsx* and *ix* in controlling female 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) except her^8. her^8 was isolated by the same method as her^3 (Pultz et al., 1994)

Molecular biology

Standard molecular biology techniques were used according to the protocols described in Sambrook et al. (1989).

Chromosome walking

DNA from the YAC clone N18-56, which has 160 kb DNA from the 36A3-13 region (Cai et al., 1994), was isolated by pulse-field gel electrophoresis. A probe was prepared from this DNA by random primer labeling and used to screen a λ library of *Drosophila* genomic DNA. Since a YAC clone of *Drosophila* DNA may contain some middle repeat sequences, wild-type fly (Canton S) genomic DNA was used to make a probe. This probe was hybridized to a set of duplicate filters of the library. Plaques identified by the YAC probe that did not hybridize to the genomic DNA probe, should contain unique sequences. 15 λ phage clones were isolated and the origin of the DNA inserts were confirmed by in situ hybridization to polytene chromosomes. Based on restriction mapping data, they form four groups (representative clones are shown in Fig. 2B, 6d2-3.4T3.2). Using the DNA inserts of selected λ clones in each group as probes, nine cosmid clones were isolated from a cosmid library of genomic DNA (provided by John Tamkun). Additional cosmid clones and λ phage clones were isolated using probes made from restriction fragments of the ends of cosmid clones to fill the gaps in the walk and

to extend the walk proximally. The isolated cosmids form a contig covering 220 kb (Fig. 2A).

Mapping of break points of deficiency chromosomes

Deficiency lines *[Df(2L)r10, Df(2L)RI1, Df(2L)RN2, Df(2L)RM5, Df(2L)TE116(R)GW16, Df(2L)TE116(R)GW18, Df(2L)H20]* (Ashburner et al., 1990) were crossed to Canton-S flies. In situ hybridization of polytene chromosome preparations of *Df(2L)/+* larvae were done according to the protocol described in Ashburner (1989), using probes made from the cosmid and λ phage clones of Fig. 2.

RFLP mapping of *her*

For RFLP mapping of *her*, two parental chromosomes were used: one carried the $cact^{F255}$ mutation which is a *P{ry⁺}* insertion line and the other the her^{3} mutation. We generated 43 recombinant chromosomes that carried both the $cact^{F255}$ and her^{3} mutations. Six RFLP markers along the walk were used and their frequencies among the recombinant chromosomes were determined by Southern analysis. A regression line was generated from these data (Fig. 2D). The intersections of the regression line with the 100% and the 0% lines of the axis that represents the percentage of the $cact^{F255}$ RFLP markers among the recombinant chromosomes defined the locations of the $cact^{F255}$ mutation and the her^{3} mutation on the walk at map positions 20 kb and 210 kb, respectively (Fig. 2D).

Genomic DNA fragments used for transformation of flies

Cosmid clone 7Q6 was digested by *Bgl*II and religated. Its insert consists of a 5′ *Not*I-*Bgl*II 1 kb fragment and a 3′ *Bgl*II-*Not*I 17 kb (B17) fragment (Fig. 2E). Since the cosmid vector is a P-element transformation vector, we were able to use this construct to transform w^{1118} flies (Rubin and Spradling, 1982; Spradling and Rubin, 1982). One of the three transformant lines (line 54A3) rescued the mutant phenotypes of all *her* allelic combinations. The 54A3 insert was mobilized by the Δ2-3 chromosome (Robertson et al., 1988) and one new line was obtained. It also rescued *her*'s mutant phenotypes. The 54A3 line is referred to as *P{her⁺}* in the text. The cosmid clone 7Q6 was digested by *Bgl*II and *Xba*I and religated to make the 10 kb B17dX transgene (Fig. 2E). None of the five transformant lines carrying the B17dX transgene rescued *her*'s mutant phenotypes. One of the inserts was mobilized by the Δ2-3 chromosome to generate 45 new lines and none of them rescued *her*'s mutant phenotypes. A 10 kb *Hpa*I fragment (H10) from 7Q6 was inserted into the pCaSpeR2 transformation vector (Pirrotta, 1988). Ten transformant lines were obtained and all of them rescued *her* mutant phenotypes (Fig. 2E).

Genomic DNA fragments used in cDNA isolation and quantitative Southern analysis

Genomic DNA fragments used for making probes are as follows (Fig. 2E). The 4R fragment is the 4 kb *Eco*RI-*Not*I end fragment of the cosmid clone 0.8N1.1. From the λ phage clone 3.4T3.2, the 3.2k fragment is a 3.2 kb *Eco*RI-*Eco*RI end fragment, the X1.4 fragment is a 1.4 kb *Xba*I-*Eco*RI fragment, the 3.5k and the 1.5k fragments are the 3.5 kb and the 1.5 kb *Eco*RI-*Eco*RI fragments, respectively. The 3.4T fragment is the 3.4 kb *Eco*RI-*Not*I end fragment of the cosmid clone 7Q6. The 7W fragments is the 7 kb *Eco*RI-*Not*I end fragment of the cosmid clone 3.4T1.1.

cDNA isolation

Probes were made from DNA fragments 4R, 3.2k, 3.5k, 1.5k and 3.4T. A larval imaginal disc cDNA library (provided by A. Cowman) was screened. Four, fifteen, four, twenty two and zero clones were isolated using the 4R, 3.2k, 3.5k, 1.5k and 3.4T probes, respectively. Representative clones from the four groups were used to make probes to hybridize Southern blots of *Eco*RI restriction digests of all of the isolated cDNA clones. Based on the hybridization data, the 45 cDNA clones form two non-overlapping classes, representing two transcription units. One cDNA clone (4Ra) representing the distal transcription unit was used to make probes to screen separate male and female third instar larval λ libraries (provided by S. Elledge) and a λ ZAP head library (DiAntonio et al., 1993). Two female-specific, seven male-specific and thirteen λ ZAP cDNA clones were isolated.

cDNA and genomic DNA sequencing

cDNA inserts were subcloned into Bluescript vectors, or rescued as plasmids from lambda ZAP clones, and sequenced by the dideoxy method using standard procedures. The genomic DNA fragments (4R and 3.2k) that contained *her* were subcloned into Bluescript vector and sequenced by the same method.

Mapping the breakpoints of the her^{8}, her^{mat} and *Df(2L)H20* mutations

The same *CyO* balancer was used to balance the *b* her^{8} *pr*, the *b* her^{3} *pr*, the *b* her^{mat} and the *Df(2L)H20*, *b pr* chromosomes. The parental chromosome from which the her^{8} and her^{3} mutations were derived is *b pr*. Genomic DNAs were isolated from adult flies of the following genotypes: *b* her^{8} *pr/CyO*, *b* her^{3} *pr/CyO*, *b* her^{mat}*/CyO*, *Df(2L)H20, b pr/CyO* and *b pr.* Southern blots of restriction digests of these genomic DNAs were made and hybridized with probes made from the DNA fragments 5.2V, 4R, 3.2k, X1.4, 3.5k, 1.5k, 3.4T and 7W. Based on the relative intensities and the sizes of the bands on the Southern blots, the breakpoints of the her^{8}, her^{mat} and the *Df(2L)H20* mutations were determined (Fig. 2E, data not shown). No genomic DNA aberrations were detected in the her^{3} mutant chromosome.

hsp-her transgene

A 1.7 kb *Eco*RI insert of cDNA clone 4Ra6 was put into *Eco*RI site of pCaSpeR-*hs* vector. The 4Ra6 clone sequence ends at nucleotide position 2094 (Fig. 3), and thus does not contain any of the polyadenylation signals described in the text. 14 transformant lines were obtained and 12 of them rescued the *her* mutant phenotypes. Heat shock was given for 30 minutes per day at 37°C for 5 days starting from 24-48 hours after egg-laying.

Northern analysis

A developmental northern blot was provided by John Hebert, which contained 20 μg of total RNA per lane. The blot was probed with a ^{32}P-labeled riboprobe which was made by in vitro transcription of *her* cDNA clone 4Ra6. 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 phosphor imaging system Molecular Imager GS363.

RT-PCR

1 μg of total RNA was reverse transcribed using random hexamers as primers. One tenth of the sample was used in a 100 μl standard PCR reaction (Innis et al., 1990). PCR conditions were as follows: 94°C, 2 minutes followed by 40 cycles of 94°C, 1 minute; 42°C, 1 minute; 72°C, 1 minute. The sequences of the primers used are:

#6=5′ GCTGAAGGAACATAAGC 3′
#25=5′ GCTGGACAGAAATTGAAGTGCTC 3′
#17=5′ CTGCCCCATAAAGAGCACTTC 3′
#12=5′ TGTCCTTGATTATCTGCAT 3′

Scanning electron micrography

Adult flies were dehydrated once in 30%, 50%, 75% and twice in 100% ethanol for at least 12 hours per concentration, followed by 30%, 50%, 70% and 100% (twice) Freon (Electron Microscopy Sciences) treatment for 12 hours at each step, air dried and gold coated using Polaron E5400 High Resolution Sputter Coater. Photos were taken using a Philips 505 SEM.

Staining of the male-specific muscles

The procedure has been previously described (Taylor, 1992) except

that here rhodamine-conjugated phalloidin (Molecular Probes) was used (10 U/ml in PBS).

RESULTS

Molecular cloning of *her*

her was previously localized to the salivary region 36A6-11 on the left arm of the second chromosome (Pultz et al., 1994). We cloned the region containing *her* by chromosome walking using cosmid and λ libraries of *Drosophila melanogaster* genomic DNA (see Materials and Methods for details). Representative cosmid and λ clones from the walk are shown in Fig. 2A,B. They cover a 220 kb region. Selected cosmids from the walk were used to make probes for in situ hybridization to localize the proximal breakpoints of five deficiencies *[Df(2L)r10, Df(2L)RI1, Df(2L)RN2, Df(2L)RM5, Df(2L)TE116(R)GW16]* that cytologically and genetically had been shown to be close to the distal side of the *her* gene, and to localize the distal breakpoint of a deficiency *[Df(2L)H20]* that is near the proximal side of the *her* gene (Fig. 2C, see Materials and Methods for details) (Ashburner et al., 1990; Pultz et al., 1994). The closest breakpoints that flank the *her* locus were the proximal breakpoints of *Df(2L)TE116(R)GW16* and *Df(2L)TE116(R)r10*, and the distal breakpoint of *Df(2L)H20*. These breakpoints turned out to be 150 kb apart. Since no other breakpoints were available to delimit *her*'s precise location in the walk, RFLP mapping (Davis and Davidson, 1984) was employed to locate *her* (see Materials and Methods for details). RFLP mapping positioned *her* approximately 10 kb distal to the breakpoint of *Df(2L)H20* (Fig. 2D).

To identify the *her* gene, genomic DNA rescue experiments were performed. Three overlapping genomic fragments in the region where RFLP analysis had located *her* were reintroduced

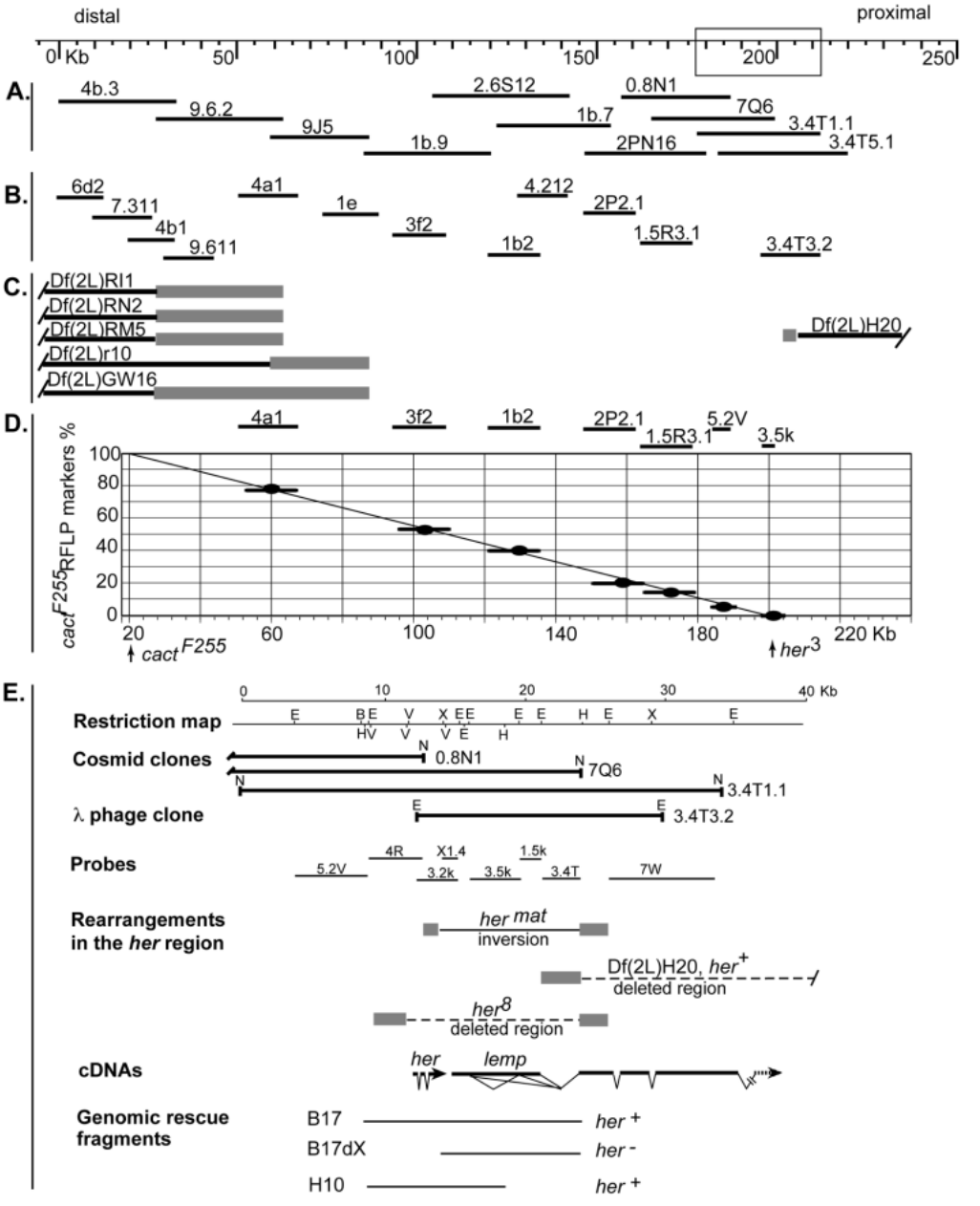

Fig. 2. Molecular cloning of the *her* gene.(A) Cosmid clones of genomic DNA. (B) λ phage clones of genomic DNA. (C) Mapping of deficiency break points. Lines represent regions deleted and shaded bars represent regions where break points are located. *Df(2L)GW16* is *Df(2L)TE116(R)GW16*. (D) RFLP mapping of her^3 (see Materials and Methods for details). At the top are λ phage clones and DNA fragments used for probes. The locations of *her* and $cact^{F255}$ are marked by arrows. (A-D) DNA regions are registered to the ruler shown at the top of A and the origin is arbitrarily chosen. The details of the region marked by a rectangle on the ruler is shown in E. (E) At the top is the ruler showing the scale and the origin is arbitrarily chosen. Beneath the ruler is the restriction map of the genomic region containing *her* and most of the gene defined by the proximal transcription unit, which is named *the last empress (lemp)*. B, *Bgl*II; E, *Eco*RI. H, *Hpa*I; N, *Not*I; X, *Xba*I; V, *Eco*RV. DNA rearrangements of *her* mutants are shown. Shaded bars represent regions where break points are located. The distal break point of her^8 is localized within a 3 kb *Eco*RV genomic DNA fragment. The proximal break point of her^8 is localized within a 2 kb *Hpa*I-*Eco*RI genomic DNA fragment. In 'cDNAs', the thick lines represent genomic locations of *her* and *lemp* cDNA sequences, the thin lines represent regions of introns and dashed line represents cDNA sequences not contained in the walk. Genomic fragments used for transformation and their activity of *her* are shown.

into flies by P-element-mediated germline transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Two of the fragments (B17 and H10) rescued all *her* mutant phenotypes and one fragment (B17dX) did not rescue any of the *her* mutant phenotypes (Fig. 2E). Using probes made from restriction fragments contained in the genomic DNA fragment B17, two non-overlapping classes of cDNA clones were isolated, representing two transcription units (Fig. 2E, see Materials and Methods for details). The distal transcriptional unit is contained in the genomic DNA fragments B17 and H10 that rescued *her* mutants and is not contained in the genomic DNA fragment B17dX that failed to rescue *her* mutants. Sequencing of a cDNA clone from the proximal transcription unit showed that its coding region lies outside of, and proximal to, the rescue fragment H10 (data not shown). Therefore, the distal transcription unit is the *her* gene.

Genomic rearrangements were observed both in the her^{8} allele (but not in its parental chromosome) and in the her^{mat} allele (Redfield, 1924) (its parental chromosome is not available). These breakpoints were mapped by quantitative Southern analysis and by detection of rearranged end fragments in genomic Southern blots (Fig. 2E). In her^{8}, the entire coding sequences of *her* and the 5′ region of the transcription unit immediately proximal to *her* are deleted (Figs 2E, 3, legend). The distal breakpoint of the deficiency *Df(2L)H20* was mapped in the same way (Fig. 2E, see Materials and Methods for details).

her encodes a protein with four atypical C_2H_2-type zinc fingers

The three longest independent *her* cDNA clones were completely sequenced. Nine additional independent *her* cDNA clones were partially sequenced. A genomic fragment named 4R (a 4 kb *Eco*RI-*Not*I end fragment of the cosmid clone 0.8N1) and a genomic fragment named 3.2k (a 3.2 kb *Eco*RI end fragment of the λ phage clone 3.4T3.2), which overlap with each other at one end, were partially sequenced (Figs 2E, 3). The sequenced portions of the 4R and 3.2k fragments contain all of the sequences of the sequenced *her* cDNA clones (Fig. 2E). All of the cDNAs have

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TTTTTTTCCTTATTCCCAATTTGTTAAAACCTCACTTTACAGCAATTTTAAAAAGTCCGC   60
GCAACATAACAGCGATTAGGAACACTGTCACTGTAATAATCTTTGCTGTACTTTTTTTGC  120
CGTAGTTGTGAATTTCGTATCCCGTGACTTTTGTTGAATAATCGATATCAACGAACAGTA  180
ATCGATATGTGCTCTAAACAAACAAACATGTAATTAAAATTAATTAAAATACGGACAGTT  240
TGTTTACCCCAAAACAGATAGTTTAGTGATCCACCGAAATGAGTGTGTTCCCAAGATATC  300
CAAACACCAGGCTAGACCTGTAGCATTAATAATATTGTGGTCTTATTGTGGACTTTTGCA  360
ATGCTTAGTGCGGATCGGGATTCGGTGGAGGAAGAATATGGATCGTGCTCGGCcTGGAAA  420
M  L  S  A  D  R  D  S  V  E  E  E  Y  G  S  C  S  A  W  K
TTCCGTTGCAGCGTGGACAGGTGCCCCTACAGGACGAACAGACCCTACAATCTGGCGCGT  480
F  R  C  S  V  D  R  C  P  Y  R  T  N  R  P  Y  N  L  A  R
CATGAGGAGTCCCACATTGGTATTACGCAGTCCAAGCTATACGGATGCCCCGTTTGCGTT  540
H  E  E  S  H  I  G  I  T  Q  S  K  L  Y  G  C  P  V  C  V
TACAACACCGACAAGGCGTCGAACCTGAAGCGGCACGTGTCTATCAAACATCCTGGGTGC  600
Y  N  T  D  K  A  S  N  L  K  R  H  V  S  I  K  H  P  G  C
AAAAAGCGCCCTCCGGAAGCGCAGCACAAGGATCGCAATGCCAAGTTGCAGTGCCTGGTG  660
K  K  R  P  P  E  A  Q  H  K  D  R  N  A  K  L  Q  C  L  V
ATGGGATGCAGATACGAGACAAATCGTCCATACGACCTCAAGCGCCACTTGATGGTGCAC  720
M  G  C  R  Y  E  T  N  R  P  Y  D  L  K  R  H  L  M  V  H
AACAATCCGGAGAAGAGCCACAGGACGTTTAAGTGCTCCCTTTGCACCTACAGCTCCGAC  780
N  N  P  E  K  S  H  R  T  F  K  C  S  L  C  T  Y  S  S  D
CGAAAGGCCAATCTCAAGCGGCACCACGAGCTACGCCATTCGGGCATAGAGGAGGCCATT  840
R  K  A  N  L  K  R  H  H  E  L  R  H  S  G  I  E  E  A  I
CAAACTGCGGAGGAACTGCGCCAGGAGATGCTGCTGGAGAAGCAAATGCTGAAGGAACAT  900
Q  T  A  E  E  L  R  Q  E  M  L  L  E  K  Q  M  L  K  E  H
AAGCTAAAATATCAGATCACAGAGAAACAGGATTTGAAGAATCTAAAGTTGAAGGACCAA  960
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K  Q  K  V  Q  M  L  R  D  Q  Q  P  K  Q  R  Q  K  K  E  Q
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K  E  A  L  V  G  S  M  K  Y  D  E  E  S  L  E  F  V  Y  E
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E  L  Q  D  E  E  Q  P  P  I  I  T  I  S  N  P  Q  I  L  H
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G  D  I  R  D  K  H  V  I
cttcaataatttacaacatttacaatgtttgcagGCTGTCAACGTTGATGGCCAGTTGCG 1320
                                  A  V  N  V  D  G  Q  L  R
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  W  F  Q  S  I  D  P  P  P  G  A  P  T  K  L  E  L  P  I  K
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CCTTGCCGAAGAAGATCAGCAGGATGAGTTTCTTGTGCCAATGCTGAGCGGAGAGGAACC 1500
  L  A  E  E  D  Q  Q  D  E  F  L  V  P  M  L  S  G  E  E  P
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  V  K  I  K  Q  S  S  L  T  D  L  A  W  S  W  S  T  P  D
aagaaagaactataaacgaaaaaaacgaacttttcctaacaatgtttttatcactaaagG 1620
                                                            A
CTGTACATCACATAAGTCCCAAAGCGGAACTCAAGAAGGACCTTAAAGCTGGCGATGCAG 1680
  V  H  H  I  S  P  K  A  E  L  K  K  D  L  K  A  G  D  A  D
ATACAGATTTTCCAGATTGGTGGGATGATGGCAAGTACACAAAGATGCAAAAGAACCAAA 1740
  T  D  F  P  D  W  W  D  D  G  K  Y  T  K  M  Q  K  N  Q  T
CATTTGCAAAGCACTCAAAGAAGCCGTCGCAGGAGAATGTGCAACGCATCCTGCGCATCA 1800
  F  A  K  H  S  K  K  P  S  Q  E  N  V  Q  R  I  L  R  I  I
TCTATGACATTTACTACAAACCCTTCAAGGAAGATCGCAAGCAGTTCGAGGCATTTCAAA 1860
  Y  D  I  Y  Y  K  P  F  K  E  D  R  K  Q  F  E  A  F  Q  I
TTAAGGACTCCTGGCTGTGCGCGACTCGAATGACTCGCATGCAGATAATCAAGGACATGT 1920
  K  D  S  W  L  C  A  T  R  M  T  R  M  Q  I  I  K  D  M  Y
ACTCTAAACAGGGCACGTTAAGTCAAACTTGATGATAAAAAATGTATCATCCCTTTGGTA 1980
  S  K  Q  G  T  L  S  Q  T  *  *  *
TTTGTATAAGAATACCATTAAGTTGTTTACTATTTAAAGTGTAAAGTAAACGAATTAAGA 2040
AAATCCCAAGTACTTTAAAACTAGTTAATTAACCTTTAATTTAAGCCCAAAAACAAGTAT 2100
AGGAGTAGTTTAAAAAAGAAAGAATACTATAGTCGAGATCCCCGACTATCATATAACCGT 2160
TACTCAGCTAGTGTGAATGCGAACGCGAAATTTAATCCTCTGGGATATTAATAAATATTG 2220
TATAATGTGAAAAAATTTAAAAAAAATTCAAACGTGTGGGCGTGACCGGTTTGGCGGCTT 2280
TAGGGCGTTGGAATGGGCGTGGCAAAAAGTTTTTTGGCAAATCGATAGAAATTTAAGCGA 2340
CTAATAAAATTATGAAAAATATCCAACAATTTTTTTTTTAAATATGGGCGTGTCAGTTTT 2400
GTGCGGTTTGTGGGCGTCGCAACATGGGTTCCGCTGCGTCTTTATCTCTAGAATATGTAT 2460
GCTAAATCTCAACCTTCTAGCTTTTAAAGTTTCTGATATCTCGTCGTTCATACGGACAGA 2520
CGGACATGACTAGATCGACTCGGCTATTGATTCTGATCAAGAATATATACTTATATATAA 2580
ATATATACACTTTATATAGTCGGAAACGCTTTCTTCTGCATGTTACATACTTTTCAACGA 2640
ATCTAGTATACCCTTTTACTCTACGAGTAACGAGtATAAATTGTGTGTTGAAAATGTCTA 2700
CACTACGATCGATTATTGAAGAAAAAATAAATTCAATGAAAATTAAAAACAATTAGTCAA 2760
TAATTATATATTATTTTAAATATGACTATTTATAGTACCGGATAATAATCATATTTAAAA 2820
AAGTGTATTTAATATTAACAGCTAAATACTGTAATGATTTTAAGCTAAATACAAATAAAA 2880
TCAGCTAAATACTGAAATCACAAATTTATGTGCCTTGTGATTTTCGACGATCGATACTTG 2940
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Fig. 3. Genomic DNA and the cDNAs sequences of the *her* gene. The conceptual protein sequences are shown in single letters. The C and H residues of the four zinc fingers are outlined. The C-terminal region is rich in N and contains two 13-mer repeats with unknown function: EKQMLKEHKLKYQ and EKQDLKNLKLKDQ (indicated by the underline). Nucleotides that are polymorphic are in lower case without an underline. The single underlined lower case letters represent intron sequences. The polyadenylation signals are double underlined. The sequences of cDNA clone 4Ra6 starts at nucleotide 238 and ends at 2094 without poly(A) sequences. It has the first intron sequences, but does not have the second intron sequences. The cDNA clone 4Rd starts at 403 and ends at 2229 with poly(A)(A_{24}). It does not contain either the first or the second intron sequences. The distal break point of her^{8} is mapped 5′ upstream of the *Eco*RV site located at nucleotide position 164 (GATATC). The GenBank accession number for the *her* genomic DNA sequence is AF025540.

the same open reading frame (ORF), but differ in the lengths of their 3′ untranslated regions (3′UTRs), because of the use of alternative polyadenylation sites (Fig. 3). Of the five cDNAs whose 3′ ends were sequenced, two have a poly(A) sequence beginning at nucleotide position 2229, two at nucleotide position 2360 and one ends at nucleotide position 2885 without a poly(A) sequence (Fig. 3). Since poly(A) addition usually occurs about 10~50 nt 3′ of the AAUAAA signal (Lamond, 1995), the AAUAAA signals at positions 2215, 2348, 2879 are probably used for poly(A) addition (Fig. 3). Comparison of the genomic and cDNA sequences revealed that the *her* gene has two small introns; the first one is 66 nt long and the second 60 nt long (Fig. 3).

The long ORF common to all sequenced cDNAs encodes a 487 amino acid protein (Fig. 3). To prove this was the HER protein, one of the completely sequenced cDNAs (4Ra6; see Fig. 3 legend) was cloned into a P element transformation vector (pCaSpeR-*hs*) containing an *hsp70* promoter and an *hsp70* 3′UTR (Pirrotta, 1988) and introduced into flies by germline transformation. This transgene *(hsp-her*) rescued all *her* mutant alleles including a null allele (*her*^{8}, Fig. 2) and thus encodes all *her* functions including maternal and zygotic, sex-specific and sex non-specific functions (Fig. 4; Table 1; the fertility of *her*^{8}*/her*^{8} females is not rescued due to a partial deletion of an adjacent gene, Fig. 2). In the absence of heat shock, the basal level of expression of the *hsp-her* transgene in *her* mutant mothers is sufficient to rescue the *her* maternal sex-specific phenotype, since *her*^{1}*/her*^{mat}*; P{hsp-her}/+* females produce equivalent numbers of daughters and sons (data not shown). Since the *hsp-her* transgene does not contain any of the *her* endogenous AAUAAA signals (see Materials and Methods), the multiple polyadenylation sites in the *her* gene are not likely to have essential regulatory functions.

The putative HER protein sequence consists of two domains, the N-terminal domain containing four C_2H_2-type zinc fingers, and the C-terminal domain which has no known structural motifs (Fig. 3). The zinc fingers in the HER protein suggest that it is likely to function as a transcription factor (for a recent review see Klug and Schwabe, 1995). However, all four of the *her* zinc fingers deviate substantially from the consensus sequences of the C_2H_2-type zinc finger motifs: E-(K/R)-P-(F/Y)-x-C-$X_{2,4}$-C-x-(K/R)-x-(F/Y)-x_5-L-x_2-H-$x_{3,4}$-H (Gibson et al., 1988; Krizek et al., 1991) (Fig. 5). Most notably, the highly conserved aromatic residue (F or Y) at position 12 is replaced by an S or a T residue and the conserved basic residue (K or R) at position 10 is replaced by an Y residue (Fig. 5).

To estimate frequencies of the HER-type changes at position 10 and 12 among known C_2H_2-type zinc finger motifs, we searched the PIR database (Release 50.0). There were 2314 $Cx_{2,4}Cx_{12}Hx_{3,4}H$ motifs in 522 polypeptides. 87%

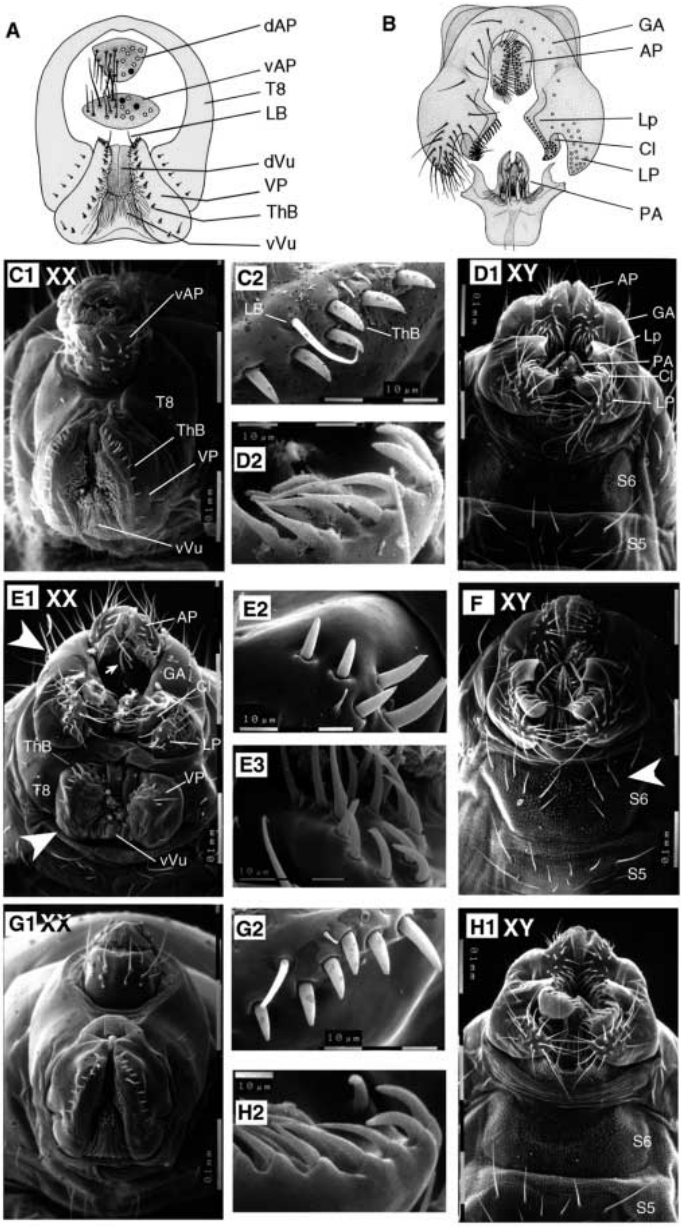

Fig. 4. *hsp-her* rescues *her* mutant phenotypes in genitalia and analia of females and in the sixth sternite of males. (A,B) Schematic drawings of the genitalia and analia of the wild-type XX female (A) and XY male (B), modified from Epper and Nöthiger (1982). Posterior is towards the top and anterior is towards the bottom. Note that anal plates are oriented dorsal-ventrally in females and laterally in males. The small circles represent the positions of the bristles. (C-H) Scanning electron micrographs of genitalia and analia of XX flies (C,E,G) and XY flies (D,F,H). Ventral view with posterior towards top. (C,D) Wild type; (E,F) *her*^{1}/*her*^{3} mutants; (G,H) *her*^{1}/*her*^{3}; *hsp-her*/+ rescued flies. (C2,E2,G2) Enlarged view of the thorn bristles of XX flies in C1,E1 and G1. (D2,H2) Enlarged view of the clasper bristles in XY flies in D1 and H1. (E3) The clasper bristles in the XX fly in E1. The dorsal anal plates are not visible in C1 and G1. In E1, the laterally oriented anal plates are fused at dorsal end, and the rudimentary ventral anal plate is indicated by the bristles (white arrow). Note that in the XX; *her*^{1}/*her*^{3} fly, both the female and male genitalia structures (indicated by large white arrowheads) are present and the anal plates are male type (E1-E3). Also note that in the XY; *her*^{1}/*her*^{3} fly, the S6 has extra bristles (F, indicated by a large white arrowhead). These mutant intersexual phenotypes are rescued by the *hsp-her* transgene (compare G1 with E1 and C1, and H1 with F and D1). Abbreviations are as follows. Female (XX): dAP, dorsal anal plate; vAP, ventral anal plate; T8, tergite 8; LB, long bristle; dVu, dorsal vulva; VP, vaginal plate; ThB, thorn bristle; vVu, ventral vulva. Male (XY): GA, genital arch; AP, anal plate; Lp, lateral process of the genital arch; Cl, Clasper; LP, lateral plate; PA, penis apparatus. S6, sternite 6 ; S5, sternite 5.

Table 1. *hsp-her* transgene rescues *her* mutant phenotypes*

Genotypes†	XX	XY	XX;*hsp-her*	XY;*hsp-her*
	semi-lethal	semi-lethal	viable	viable
her^1/her^1	intersex	intersex	female	male
	sterile	fertile	fertile	fertile
	semi-lethal	semi-lethal	viable	viable
her^3/her^3	-	-	female	male
	-	-	fertile	fertile
	lethal	lethal	viable	viable
her^8/her^8	-	-	female	male
	-	-	sterile‡	fertile
	lethal	lethal	viable	viable
her^3/her^8	-	-	female	male
	-	-	fertile	fertile
	viable	viable	viable	viable
her^{mat}/her^{mat}	female	male	female	male
	semi-sterile	fertile	fertile	fertile
	daughterless		daughters-viable	

*All flies were raised at 25°C except that flies carrying the *hsp-her* transgene were heat shocked (see Materials and methods for details).

†All chromosomes that contain *her* mutations are marked by *b*.

‡The fertility of her^8/her^8 females is not rescued due to a partial deletion of the adjacent gene *lemp* (Fig. 2).

(2022/2314) of them have an F or Y at position 12 and 78% (1799/2314) have a K or R at position 10. Only 3.4% (78/2314) were HER-type motifs having both a Y and a S/T at position 10 and 12, respectively [$Cx_{2,4}CxYx(S/T)x_8Hx_{3,4}H$]. Remarkably, all except 8 of the 78 HER-type zinc fingers are encoded by the X and Y chromosome genes *Zinc Finger X (ZFX*) and *Zinc Finger Y (ZFY*) and their homologs from frog, alligator, chicken, mouse and human (their protein products are referred to as ZFY-like proteins hereafter). These fingers correspond to the even-numbered fingers of the ZFY-like proteins (the fingers are numbered according to their relative positions in a protein) (Fig. 5; Dilella et al., 1990). Detailed structural studies have shown that the residue substitutions in ZFY even-numbered fingers retain the ββα secondary structural motif common to most C_2H_2 zinc fingers with known three-dimensional structures (Kochoyan et al., 1991). However, they do alter internal architecture and surface topology relevant to the putative DNA contacting surface (Kochoyan et al., 1991). Another feature shared by the zinc fingers of HER and ZFY-like proteins is a pairwise repeat pattern such that odd-numbered or even-numbered fingers are more similar to each other than are odd-numbered fingers with even-numbered fingers (Fig. 5). No significant sequence similarity was found between HER and ZFY-like proteins outside the zinc fingers.

Besides the zinc fingers of ZFY-like proteins, there are eight other HER-type zinc fingers in the PIR database. Five of them are from the human REST protein (nine zinc fingers in toto) (Chong et al., 1995), two are from the chicken CTCF protein (eleven zinc fingers in toto) (Filippova et al., 1996; Klenova et al., 1993) and one from the sea urchin P3A1 protein (two zinc fingers in toto) (Zeller et al., 1995). All three of the proteins are transcription factors with sequence-specific DNA binding activity.

Besides the zinc fingers, the HER protein sequence does not have significant sequence similarity to proteins in the public databases that we have searched.

her is transcribed throughout all developmental stages and in adults of both sexes

To gain insight into how *her* is regulated, we monitored the levels of *her* transcripts. Northern analysis shows that *her* is transcribed throughout development (Fig. 6A,B). The high level of transcripts in 0-2 hour early embryos are mostly maternal, since zygotic transcription is negligible in 0-1.5 hour embryos (Anderson and Lengyel, 1981). The level of transcripts drops substantially during 2-4 hours of embryo development, indicating a lag between loss of maternal transcripts and the synthesis of zygotic transcripts. There is a higher level of *her* expression during 4-8 hours of embryo development. After about 8 hours of embryo development, *her* is transcribed at a low level. The patterns of *her* transcripts are similar in both sexes of adults. The level of *her* transcripts in adult females is slightly higher (less than two-fold) than in males, most likely due to maternal transcription in ovaries (Fig. 6B). The northern analysis data, together with our result that the *hsp-her* transgene completely rescues *her* mutations, strongly suggest that there is no sex-specific regulation of *her* at the level of transcription.

These northern results also show that, although there are four polyadenylation signals in the *her* 3′UTR, the frequencies of their usage are different. The polyadenylation signals at nucleotide positions 2731 and 2779 are not used frequently because the sizes of these predicted transcripts would be at least 2.470 kb while the sizes of the majority of the *her* transcripts on the northern blot fall between 1.9 kb and 2.2 kb (Fig. 6B).

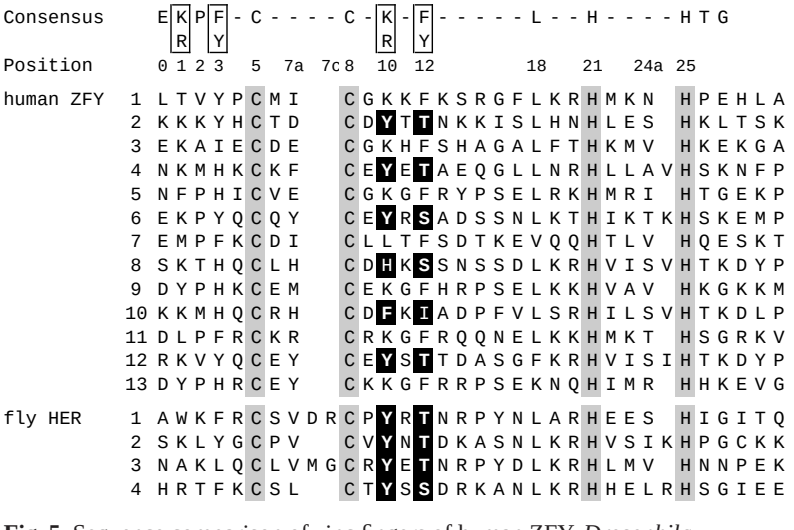

Fig. 5. Sequence comparison of zinc fingers of human ZFY, *Drosophila melanogaster* HER proteins. All zinc fingers of human ZFY and fly HER are shown. The conserved C residues at position 5 and 8 and the conserved H residues at position 21 and 25 of the zinc fingers are shown in gray background. The atypical amino acid residues at position 10 and 12 are shown in black background. In mouse ZFY-1 and ZFY-2, the amino acid residue at position 12 of finger 10 is an S, rather than an I.

Splicing of *her* introns occurs in both sexes

Since much of the regulation in the *Drosophila* somatic sex determination hierarchy occurs by sex-specific alternative splicing, one possibility was that *her* might also be controlled at this level. Because the two introns in *her* pre-mRNA are small (66 nt and 60 nt), we could not ascertain whether *her* is regulated at the level of splicing based on the northern results. The splicing of the second intron of *her* is unlikely to be regulated, since the *hsp-her* construct does not contain the second intron and rescues *her* mutations in both sexes. However, the *hsp-her* construct does contain the first intron. We therefore used an RT-PCR assay to examine splicing. Since the alternative splicing of *Sxl*, *tra*, *dsx* and *fru* pre-mRNAs are readily seen in adults (Horabin and Schedl, 1993; Nagoshi et al., 1988; Ryner et al., 1996), we focused on the analysis of *her* in adult tissues. Both introns of *her* pre-mRNAs are spliced in adult males and females, demonstrating that there is no sex-specific splicing of the *her* pre-mRNA in the majority of adult tissues (Fig. 6C,D). Consistent with this result, partial sequencing of two cDNA clones, one isolated from a male cDNA library and the other from a female cDNA library, showed that the clones have both introns spliced and use the same splice sites (see Materials and Methods; data not shown). These data thus indicate that *her* is not regulated at the level of splicing by any of the sex determination genes that are expressed sex-specifically (*Sxl*, *tra* and *dsx*). This conclusion was confirmed in the case of *tra* by the finding that both *her* introns are spliced in XX; *tra /tra* and XY; *tra /tra* flies (Fig. 6C,D). The RT-PCR assay also showed that both *her* introns are spliced in *ix* mutant XX and XY flies, demonstrating that *ix* is not involved in *her* pre-mRNA splicing (Fig. 6C,D). In addition, we showed that *tra-2* does not control *her* expression at the level of splicing (Fig. 6C,D).

ix does not control *her* expression

With respect to the regulation of *her* expression, the available data did not rule out the possibility that *her* transcription is regulated by the *ix* gene in sexual dimorphic tissues. If *ix* had all of its effects on sexual differentiation through transcriptional regulation of *her*, then the *hsp-her* transgene should completely rescue the mutant phenotypes of *ix* flies since the transgene encodes all of *her* functions and constitutively expresses *her* mRNAs. To test this possibility, we crossed a chromosome that carries the *hsp-her* transgene into *ix* mutant flies and examined whether the *hsp-her* transgene was able to rescue the adult cuticle phenotypes of the XX; *ix* mutant flies. We used two independent transformant lines that contain the *hsp-her* transgene and have levels of *her* expression that rescue the *her* mutant phenotypes. The *hsp-her* transgenes had no effect on the adult cuticular phenotypes of the XX; *ix* mutant flies (data not shown), demonstrating that *ix* does not exert any significant part of its sex differentiation functions through transcriptional regulation of *her*.

MOL development is normal in *her* mutants

The somatic sex differentiation pathway has two branches downstream of *tra* and *tra-2* represented by the *dsx* gene and the CNS-specific *fru* gene, respectively (see Introduction). All known *her* zygotic functions are in conjunction with the *dsx* branch (see above). However, the question of whether *her* also functions in the *fru* branch has not been addressed previously. *fru* is known to be required for the development of a male-specific abdominal muscle termed the Muscle of Lawrence

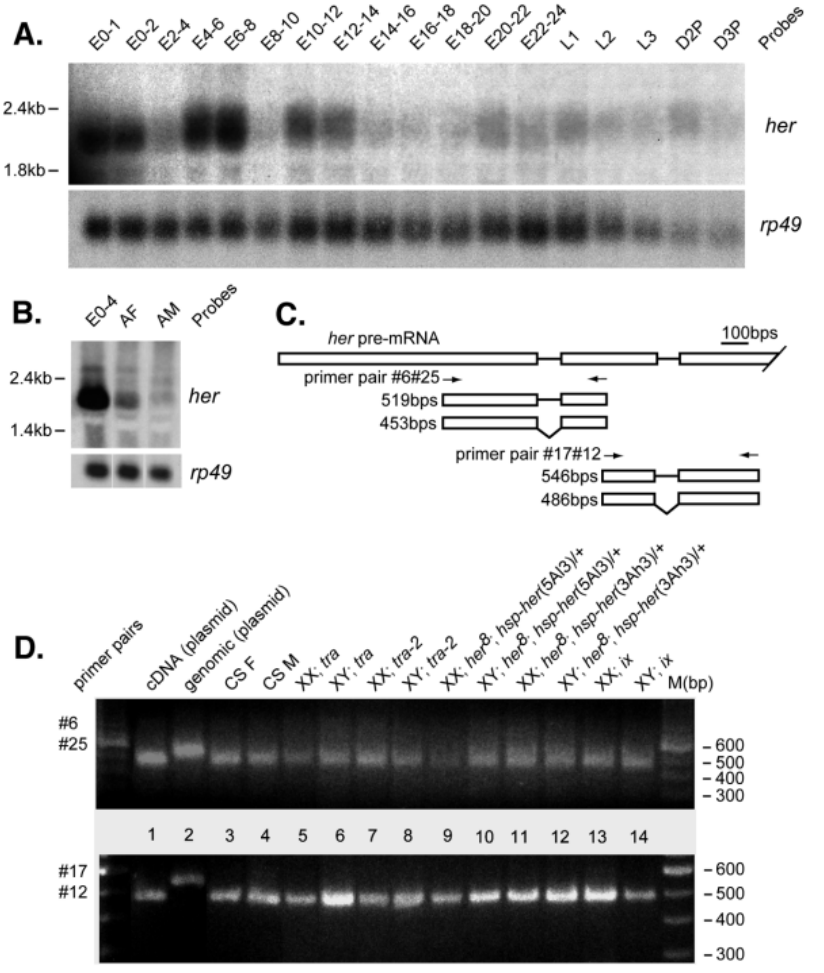

Fig. 6. The transcription and splicing of the *her* transcripts are not sex-specifically regulated. (A,B) Developmental northern blots of the *her* transcripts. Total RNAs are used in A and poly(A)$^+$ RNAs are used in B. The Northern blots were hybridized with probes made from an *her* cDNA and from *rp49* genomic DNA (see Materials and methods for details). E0-1, 0-1 hour embryos; L1, first instar larvae; D2P, 2-day-old pupae; AF, adult female; AM, adult male. Note that the *her* transcripts are present in all developmental stages and in both sexes of adults. (C) Positions of the RT-PCR primer pairs and the expected sizes of the RT-PCR products from the *her* transcripts. (D) Shown are the *her* RT-PCR products in agarose gel electrophoresis from total RNAs of adult flies of various genotypes (see Materials and methods for details). As the size controls, lanes 1 and 2 show PCR products using *her* cDNA clone 4Rd (Fig. 3) and genomic DNA clone 3.2k (Fig. 2) as templates, respectively. CS F, Canton-S female; CS M, Canton-S male. In lanes 5-8, Y is B^sY and *tra-2* is *tra-2*^{B}. In lanes 10-13, 5Al3 and 3Ah3 represent two independent chromosomes carrying the *hsp-her* transgene (see text).

(MOL) in males; but it is unclear whether it is required for MOL's absence in females (Gailey et al., 1991; Ryner et al., 1996). We used the presence/absence of the MOL to assess the possible role of *her* in the *fru* branch. her^1/her^3 is one of the strongest mutant allelic combination that still gives viable adults at 25°C. The MOLs were still present in her^1/her^3 mutant males and absent in females whose muscles are indistinguishable from those of wild-type females (data not shown). This result indicates that *her* is neither required in males for the MOL development, nor required in females for the absence of the MOL development. Moreover, expression of *her* using the *hsp-her* transgene has no effects on the development of MOL in females and males (data not shown). Based on these results, we conclude that *her* does not function in MOL development and therefore is unlikely to be functioning in the *fru* branch.

DISCUSSION

On the position of *her* in the somatic sex determination hierarchy

With our current knowledge regarding *her*, how *her* functions in sex determination can most profitably be addressed by considering two topics: first, whether *her* is expressed sex-specifically or sex non-specifically, and second, where *her* functions relative to the other sex determination genes.

With respect to the first of these topics, we note that *her* cannot be responding directly to the X:A ratio only in females and functioning in parallel with *Sxl*, since the ectopic expression of *tra* in males (XY) transform them into phenotypic females, rather than the intersexes that would be expected if *her* function was absent in males (McKeown et al., 1988). These data also show that *her* cannot be functioning in parallel with *tra*, under the control of *Sxl*, since in those *tra*-expressing males, *Sxl* is not expressed and yet they are phenotypic females.

This leaves open the possibilities that *her* is expressed (1) sex non-specifically, or (2) sex-specifically in the *tra-dsx* branch of the hierarchy. If it is assumed that the TRA and TRA-2 proteins do not have biochemical functions other than their known activities as RNA-processing factors**,** then *her* cannot be a direct target of *tra* or *tra-2*, since we have shown that *her* is not regulated sex-specifically at the RNA-processing level and the splicing of *her* pre-mRNA is not affected in *tra-2* mutants. Moreover, *her* cannot be indirectly regulated by *tra* at either the translational or post-translational levels, since HER is capable of activating the expression of the *yp* genes in males in the absence of the repression exerted by DSX^M (Li and Baker, unpublished data) and, in those males, *tra* is not expressed. In addition, if it is assumed that the DSX proteins only act as sex-specific transcription factors, then *her* cannot be a direct target of *dsx*, since we have shown that *her* is not regulated sex-specifically at the transcriptional level. Indeed it seems unlikely that *her* is regulated by *dsx* at any level, since (1) *her* activates the expression of the *yp* genes in the absence of DSX^F (Li and Baker, unpublished data), and (2) *dsx* regulates the *yp* genes directly, making it very unlikely that *dsx* also regulates the *yp* genes indirectly through *her*. Finally, *her* cannot be regulated by *ix* at the level of transcription or splicing, since (1) an *hsp70-her* transgene does not ameliorate the phenotype of *ix* mutants, and (2) *ix* mutants do not affect the splicing of *her* pre-mRNA. Taken together, these considerations suggest that the most likely alternative compatible with the data is that *her* is expressed sex non-specifically.

With respect to the question of where *her* functions relative to the other sex determination genes, previous work suggested, based on the observations that zygotic *her* function does not control the transcription or splicing of either *Sxl*, *tra* or *dsx*, that *her's* zygotic function was independent of the other genes in the sex determination hierarchy and that *her*'s zygotic function might be to control sexual differentiation in conjunction with *dsx* (Pultz and Baker, 1995; Pultz et al., 1994). Our findings are entirely consistent with these suggestions. In particular, we have shown that *her* is a positive regulator of the *yp* genes and that HER acts to control the expression of the *yp* genes through regulatory sequences that are distinct from those through which the DSX proteins regulate the *yp* genes (Li and Baker, unpublished data).

On the maternal and sex non-specific functions of *her*

Genetic studies have shown that *her* has both maternal and zygotic sex-specific functions, as well as maternal and zygotic sex non-specific essential functions (Pultz and Baker, 1995; Pultz et al., 1994). Our results strongly suggest that the multiple roles of *her* represent the functioning of a single HER protein. We infer that *her*'s different functions are due to spatial and/or temporal controls of either HER's activity, or the activities of factors that interact with HER, or its targets. This is not a unique property of *her,* since, for example, the *sisterless-b* (*sis-b*) gene also encodes a single protein which is necessary for the activation of the *Sxl* early promoter and for the development of the peripheral nervous system, depending on when SIS-B is expressed and what factors it interacts with (reviewed by Cline and Meyer, 1996; Villares and Cabrera, 1987).

Two classes of genes in the sex determination hierarchy

Our studies of *her* reinforce the view that there are two classes of genes in the somatic sex determination hierarchy. The first class of genes are either sex-specifically expressed, such as *Sxl*, *tra*, *fru* and *dsx*, or expressed at higher levels in one sex than the other, such as the X-linked zygotic activators of *Sxl [sisterless-a* (*sis-a*), *sis-b*, *sisterless-c* (*sis-c*) and *runt* (*run*)*]* (reviewed by Cline and Meyer, 1996). This class of genes plays instructional roles in sex determination and differentiation. The second class of genes are sex non-specifically expressed, such as *tra-2*, the genes that act to facilitate *Sxl* auto-regulation *[sans fille* (*snf*), *fl(2)d* and *vir]* and the maternal or autosome-linked zygotic regulators of *Sxl [daughterless* (*da*), *extra machrochaetae* (*emc*), *groucho* (*gro*) and *deadpan* (*dpn*)*]*. These genes play permissive roles in sex determination and differentiation. Our results indicating that *her* is expressed sex non-specifically place *her* in the latter group. Most of the genes in the first class and all of the genes in the second class have functions other than sex determination and differentiation. Thus, only three (*Sxl*, *tra* and *dsx*) of the genes known to be required for sex determination act exclusively in sex determination and/or differentiation, supporting the view that

genes participating exclusively in one specific developmental process are rare (Thaker and Kankel, 1992).

Among the genes that are expressed sex non-specifically and play permissive roles in sex determination, there are substantive differences in the nature of their involvement in sex determination. One subset of these genes appears to have rather specific, dedicated roles in sex determination. By this we mean that these genes, examples of which are *tra-2, emc* and *gro*, function as specific co-factors for particular steps in the sex determination hierarchy, but do not appear to have broad roles as components of the general cellular machinery concerned with gene expression. For example, the only known functions of *tra-2* outside of female somatic sex determination are in the male germline (reviewed by Cline and Meyer, 1996). A second subset of these genes appears to encode general cellular functions. This type is represented by the *snf* gene, which encodes the U1A/U2B″ protein, a general splicing factor that is an evolutionarily conserved component of the spliceosome (for an extensive discussion of *snf*, see Cline and Meyer, 1996). Since *her* is also expressed sex non-specifically and has a permissive function in sex determination and differentiation, the question arises as to whether HER is a general cellular factor, or has a more restricted function. Two pieces of data point towards *her* not being a general cellular factor. First, an expected phenotype of a null mutation of many, if not most, genes encoding general cellular factors is cell lethality. Yet mitotic clones that are homozygous for a null *her* mutation are readily obtained in most, if not all, external tissues (Li and Baker, unpublished data). Second, under conditions where homozygous *her* females have good viability they exhibit strong sexual transformations (Pultz et al., 1994).

The HER protein is likely to be a transcription factor

We have shown that the *her* gene encodes a protein with four C_2H_2-type zinc fingers. The HER protein is likely to be a transcription factor, since the most common role of the C_2H_2-type zinc fingers is to serve as DNA-binding domains within transcription factors (reviewed by Klug and Schwabe, 1995). This is consistent with the prediction from genetic studies that HER might function as a transcriptional regulator, since the two distinct places (upstream of *Sxl* and either parallel to, or downstream of, *dsx*) in the somatic sex determination hierarchy where it seemed most likely that *her* acted involve transcriptional regulation (Pultz and Baker, 1995). The similarity of *her* zinc fingers to some of the zinc fingers of the REST (Chong et al., 1995), CTCF (Filippova et al., 1996) and the P3A1 (Zeller et al., 1995) proteins, which are known DNA-binding factors, suggests that *her* is a DNA-binding protein.

It is intriguing that the atypical zinc fingers of HER resemble those of ZFY-like proteins. It is unclear whether the ZFY-like proteins are involved in sex determination in mammals. The *Sex determining Region Y gene* (*Sry*) has been found to be responsible for the determination of maleness in humans and mice (reviewed by Goodfellow and Lovell-Badge, 1993; Schafer, 1995). That there might be functional relationship between the mouse *Sry* and *Zfy* genes is suggested by the correlation between the narrow spatial and temporal expression patterns in mouse genital ridge of the *Sry* gene and a *Zfy*-1::*lacZ* transgene (Zambrowicz et al., 1994). Among many possibilities, one is that the ZFY-like proteins may provide some permissive functions for the action of the SRY protein in sex determination in mammals. However, it is currently unclear whether the temporal and spatial expression patterns of *Sry* and *Zfy* in the embryonic genital ridge are correlated in other vertebrate species. It will be of great interest to know if there exist similar target sequences or interacting factors for the HER and the ZFY-like proteins in *Drosophila* and vertebrates, respectively.

In summary, our molecular characterization of *her* shows that *her* encodes a C_2H_2-type zinc finger protein, and that the expression of *her* is not sex-specifically regulated. Our findings also indicate that *her* is expressed independent of the known members of the sex determination pathway, and that *her* functions with the *dsx*, but not the *fru*, branch of the sex determination hierarchy of *Drosophila*.

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