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intersex, a gene required for female sexual development in *Drosophila*, is expressed in both sexes and functions together with *doublesex* to regulate terminal differentiation

Carrie M. Garrett-Engele[1,2,*,‡], Mark L. Siegal[1,‡,§], Devanand S. Manoli[1], Byron C. Williams[3], Hao Li[1,†] and Bruce S. Baker[1]

[1]Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, USA
[2]Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA
[3]Department of Molecular Biology and Genetics, Biotechnology Building, Cornell University, Ithaca, NY 14853-2703, USA
*Present address: Howard Hughes Medical Institute and Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA
†Present address: Department of Functional Genomics, Novartis Pharmaceuticals, 556 Morris Avenue, Summit, NJ 07901, USA
‡These authors contributed equally to this work
§Author for correspondence (e-mail: mlsiegal@stanford.edu)

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SUMMARY

Previous genetic studies indicated *intersex* (*ix*) functions only in females and that it acts near the end of the sex determination hierarchy to control somatic sexual differentiation in *Drosophila melanogaster*. We have cloned *ix* and characterized its function genetically, molecularly and biochemically. The *ix* pre-mRNA is not spliced, and *ix* mRNA is produced in both sexes. The *ix* gene encodes a 188 amino acid protein, which has a sequence similar to mammalian proteins thought to function as transcriptional activators, and a *Caenorhabditis elegans* protein that is thought to function as a transcription factor. Bringing together the facts that (1) the *ix* phenotype is female-specific and (2) functions at the end of the sex determination hierarchy, yet (3) is expressed sex non-specifically and appears likely to encode a transcription factor with no known DNA-binding domain, leads to the inference that *ix* may require the female-specific protein product of the *doublesex* (*dsx*) gene in order to function. Consistent with this inference, we find that for all sexually dimorphic cuticular structures examined, *ix* and *dsx* are dependent on each other to promote female differentiation. This dependent relationship also holds for the only known direct target of *dsx*, the Yolk protein (Yp) genes. Using yeast 2-hybrid assay, immunoprecipitation of recombinant tagged IX and DSX proteins from *Drosophila* S2 cell extracts, and gel shifts with the tagged IX and DSX^F proteins, we demonstrate that IX interacts with DSX^F, but not DSX^M. Taken together, the above findings strongly suggest that IX and DSX^F function in a complex, in which IX acts as a transcriptional co-factor for the DNA-binding DSX^F.

Key words: *Drosophila*, *doublesex*, *hermaphrodite*, *intersex*, Sex determination

INTRODUCTION

A single, multi-branched regulatory hierarchy controls all aspects of somatic sexual differentiation in *D. melanogaster* (Fig. 1) (reviewed by Cline and Meyer, 1996; Marín and Baker, 1998). This hierarchy functions, via a cascade of alternative pre-mRNA splicing steps, to generate the sex-specific products of the *doublesex* (*dsx*) and *fruitless* (*fru*) genes, which head two parallel branches. Here, we are concerned with the *dsx* branch of the sex hierarchy. Wild-type *dsx* function is necessary for all somatic sexual development outside the central nervous system (CNS) in males and females (Baker and Ridge, 1980), as well as some aspects of sexual development in the CNS (Jallon et al., 1988; Taylor and Truman, 1992; Villella and Hall, 1996). The regulated splicing of the *dsx* pre-mRNA in females results in the production of a female-specific mRNA that encodes DSX^F, whereas in males default splicing of the *dsx* pre-mRNA generates a male-specific mRNA which encodes DSX^M. DSX^M and DSX^F are zinc-finger transcription factors with identical DNA-binding domains, but different C termini (Burtis and Baker, 1989; Burtis et al., 1991; Erdman and Burtis, 1993). The *dsx* gene is the last sex determination regulatory gene in its branch of the hierarchy, as its proteins bind to, and regulate the transcription of, the Yolk *protein 1* (*Yp1*) gene (Burtis et al., 1991; Coschigano and Wensink, 1993). Functioning together with *dsx* in females are the *intersex* (*ix*) (Baker and Ridge, 1980; Chase and Baker, 1995) and *hermaphrodite* (*her*) (Li and Baker, 1998a; Li and Baker, 1998b; Pultz and Baker, 1995; Pultz et al., 1994) genes.

Previous studies have provided some insights into the functional relationships of the *her* and *ix* genes to *dsx* (Baker and Ridge, 1980; Pultz and Baker, 1995). The *her* gene is required maternally for the initial expression of the *Sex-lethal* (*Sxl*) gene at the top of the sex determination hierarchy, and in

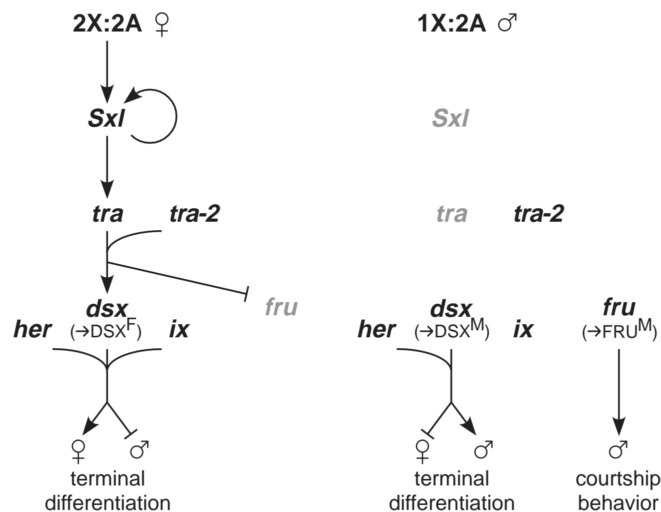

Fig. 1. The *Drosophila* somatic sex-determination hierarchy. The ratio of X chromosomes to sets of autosomes determines the on/off state of the *Sex-lethal* (*Sxl*) gene. In females, where the X:A ratio is 1, active SXL protein is made and its production is maintained via autoregulation. The presence of SXL causes splicing of the *transformer* (*tra*) pre-mRNA such that active TRA protein is made. When TRA is present with the protein product of the *transformer-2* (*tra-2*) gene, the pre-mRNA of the *doublesex* (*dsx*) gene is spliced into its female-specific form, which encodes the DSX^F protein. Similarly, the pre-mRNAs from the 5′-most promoter of the *fruitless* (*fru*) gene are spliced in a female-specific manner, and do not produce any detectable protein (three other promoters of *fru* produce transcripts that do not differ between the sexes). DSX^F interacts with the products of the *hermaphrodite* (*her*) and *intersex* (*ix*) genes to activate female terminal differentiation and to repress male terminal differentiation. In males, where the X:A ratio is 0.5, no active SXL is made, so the *tra* pre-mRNA is spliced into its default, male-specific form, which does not produce active TRA protein. Although it is present in males, TRA-2 cannot act without active TRA, so the *dsx* and *fru* pre-mRNAs are spliced into default, male-specific forms. The male-specific DSX^M protein activates male terminal differentiation and represses female terminal differentiation, interacting to some extent with HER. Although *ix* is expressed in males, like *tra-2* it has no detectable function. The male-specific FRU^M protein activates male courtship behavior. Arrows indicate positive regulation, bars indicate negative regulation and gray shading of gene names indicates that active proteins are not produced in the given sex.

addition is required zygotically for female somatic sexual differentiation and some aspects of male somatic sexual differentiation (Li and Baker, 1998a; Li and Baker, 1998b; Pultz et al., 1994). Furthermore, epistasis analysis places the zygotic function of the *her* gene in parallel to, or downstream of, the *dsx* gene (Li and Baker, 1998b; Pultz and Baker, 1995). The *ix* gene is required for female, but not male, somatic sexual development (Baker and Ridge, 1980; Chase and Baker, 1995). Genetic epistasis studies indicate that *ix* also acts in parallel to, or downstream of, *dsx* in the sex-determination hierarchy (Baker and Ridge, 1980). Moreover, molecular data indicate that neither *ix* nor *her* is required for the sex-specific splicing of *dsx* pre-mRNA (Nagoshi et al., 1988; Pultz and Baker, 1995). Therefore, the genetic and molecular data suggest that *ix*, *her* and *dsx* function at, or near, the end of the hierarchy to regulate the terminal differentiation genes in females.

Comparisons of the phenotypes of *her*, *dsx* double mutant flies with those of flies that are mutant at just one of these genes showed that *her* and *dsx* act independently to regulate some aspects of sexual differentiation and function interdependently to control other aspects of sexual differentiation in females. Thus, the DSX^F and HER proteins independently activate Yolk protein (Yp) gene expression in females. They also independently promote development of the vaginal teeth and anal plates in females (Li and Baker, 1998b). However, these proteins function interdependently to regulate female-specific differentiation of foreleg bristles and pigmentation of tergites 5 and 6 (Li and Baker, 1998b). The effect of *her* on Yp gene expression is not through the fat body element (FBE), to which the DSX proteins bind (Burtis et al., 1991; Coschigano and Wensink, 1993), but rather through Yp DNA sequences outside the FBE, consistent with the finding that these proteins control the Yp genes in an independent manner. That HER and DSX^F act independently in regulating some aspects of sex and interdependently with respect to other aspects of sex could be due to different organizations of the regulatory elements of the genes being controlled in these tissues, or to differences between the arrays of other factors regulating these genes together with HER and DSX^F.

There are also previous genetic data bearing on the relationship between *dsx* and *ix*. First, it has been reported that simultaneous heterozygosity for specific mutant alleles of *ix* and *dsx* in diplo-X flies results in a cold-sensitive intersexual phenotype (S. E. Erdman, PhD thesis, University of California at Davis, 1994) (Erdman et al., 1996). As cold-sensitive nonallelic noncomplementation is frequently indicative of protein-protein interactions (Hays et al., 1989; Stearns and Botstein, 1988), it has been suggested that there may be a physical interaction between the IX and DSX proteins. Second, it has been shown that a dsx^F transgene promotes female differentiation in an XY individual that is otherwise wild type, but not in an XY individual lacking *ix* function (Waterbury et al., 1999). These findings led Waterbury et al. (Waterbury et al., 1999) to suggest that *ix* and *dsx* function interdependently and that IX is either constitutively expressed (and therefore present in males), or directly under the control of DSX^F.

To understand how the female-specific function of the *ix* gene is established and how *ix* regulates terminal differentiation in females, we have cloned the *ix* gene. *ix* was localized to the cytological region 47F by complementation with deficiencies and further localized to a 65-kb region by restriction fragment length polymorphism (RFLP) mapping. A clone containing the *ix* gene was identified by its ability to rescue *ix* mutant phenotypes when introduced into flies by *P*-element-mediated germline transformation. The *ix* protein has sequence similarity to proteins proposed to act as transcriptional activators, but does not contain a known DNA-binding domain. Additionally, the *ix* pre-mRNA is not alternatively spliced, suggesting that the *ix* protein is present in both sexes and may interact with one or more female-specific proteins to regulate female differentiation. As IX and DSX^F are proposed to act at the bottom of the sex determination hierarchy, the possibility that these proteins cooperate to regulate female terminal differentiation genes was investigated. Analysis of females mutant for *ix*, *dsx*, or both, demonstrated that IX and DSX^F function interdependently to activate Yp gene expression and to regulate differentiation of vaginal teeth, anal plates, foreleg bristles and sixth-tergite

pigmentation. Therefore, unlike the DSX^F and HER proteins, which cooperate to control some terminal differentiation genes and function independently to regulate others, IX and DSX^F function together to control somatic sex differentiation in all female structures analyzed. A possible mechanism for the interdependence of IX and DSX^F is revealed by our demonstrations that IX interacts with DSX^F, but not DSX^M, in yeast 2-hybrid and co-immunoprecipitation assays, and that IX and DSX^F form a DNA-binding complex, as assayed by gel shift.

MATERIALS AND METHODS

Drosophila stocks

Mutations and chromosomes not referenced are described elsewhere (Lindsley and Zimm, 1992). Crosses were carried out at 25°C unless another temperature is indicated.

Polytene chromosome analysis

Deficiency breakpoints were analyzed in polytene larval salivary gland chromosomes dissected in 0.7% NaCl and stained with orcein (Ashburner, 1989). The deficiency stocks *Df(2R)17* and *Df(2R)27* (gift of R. Burgess) were crossed to wild-type flies and the *Df/+* chromosomes were analyzed. The distal breakpoints for each deficiency were determined. The insertion sites of the *P* elements #4412 and #13403 (Torok et al., 1993) were confirmed by in situ hybridization to polytene chromosomes following a standard protocol (Ashburner, 1989). Two changes to the procedure were made: the chromosomes were dissected in 0.7% NaCl and the acetylation step was skipped.

Southern analysis

Genomic DNA was isolated, electrophoresed, transferred and probed using standard techniques (Sambrook et al., 1989).

Restriction fragment length polymorphism (RFLP) mapping of *intersex*

To localize *ix* by RFLP mapping, pairs of closely linked markers flanking the *ix* locus were employed. The *P* elements $P[w^+]4412$, inserted at 47D, and $P[w^+]13403$, inserted at 48A, were used (Torok et al., 1993). To generate recombination events proximal or distal to the ix^2 mutation, *w/w;* $P[w^+]4412\ ix^2/CyO$ females were crossed to *w*; $P[w^+]13403/CyO$ males, and the Cy^+ female progeny (*w/w;* $P[w^+]4412\ ix^2/P[w+]13403$) were collected as virgins and crossed to *w; Sp/CyO; Sb/TM2* males. The male progeny of the latter cross were scored by eye color. Males with white eyes (no *P* element) and males with darker eye pigmentation (two *P* elements) were crossed to *w/w; Sp/CyO; Sb/TM2* virgin females to establish stocks of the recombinant chromosomes. To determine whether the recombinant chromosomes carried ix^2, and thereby to determine the location of the crossover relative to the *ix* locus, males carrying the recombinant chromosomes were crossed to *w/w;* ix^2/CyO virgin females.

DNA samples isolated from $P[w^+]4412\ ix^2/CyO$ and $P[w^+]1340/CyO$ flies were digested with 24 restriction enzymes and probed with DNA fragments from the *ix* chromosomal walk (Fig. 2B) to identify RFLPs between the two parental chromosomes. DNA samples isolated from the fly stocks established for 22 recombinant chromosomes balanced with the *CyO* chromosome were then analyzed using the restriction enzymes and DNA probes that identified RFLPs. This analysis indicated that six crossover events mapped proximal and 16 crossovers mapped distal to the ix^2 mutation.

DNA polymorphisms between the parental chromosomes were identified and used to analyze the recombinant chromosomes. Southern analysis with four DNA fragments (R16, 2G, 4C, P6.1) distributed across the 100 kb DNA walk (Fig. 2B) detected DNA polymorphisms between the $P[w^+]4412\ ix^2$ and $P[w^+]13403$ parental chromosomes. All six crossover events proximal to *ix* were also proximal to the R16 fragment, located within 5 kb of the *Df(2R)27* breakpoint, which indicated that all of the proximal recombination events isolated fail to further localize *ix*.

Analysis of the distal crossovers was more informative. Using the 4C and P6.1 DNA clones as probes detected 3 recombination events proximal to these probes, and the remaining crossovers occurred distal to these fragments. Results with the 4DXR DNA fragment as a probe demonstrated that all three of the crossovers proximal to phage 4C are distal to this probe. Unfortunately, because of the uneven distribution of recombination events, the location of *ix* cannot be ascertained by regression of crossover frequency on a physical map. However, the results of this RFLP analysis further localized *ix* to the region proximal to the 4C phage clone and distal to the *Df(2R)27* breakpoint, a 65 kb region.

Northern analysis

Wild-type (Canton-S) polyA+ RNA (5 μg per lane) from females and males was electrophoresed on a 1% agarose/1.85% formaldehyde gel, then transferred to a Hybond-N+ membrane and fixed by alkali treatment. For sizing bands detected by autoradiography, an RNA ladder (Life Technologies, Rockville, MD) was also run on the gel and visualized by staining with ethidium bromide. Hybridization with an antisense *ix* probe labeled with [α-^{32}P]UTP was carried out overnight in 5×SSPE, 5×Denhardt's solution, 0.4% SDS, 10 μg/ml salmon sperm DNA, 50% formamide at 60°C. Two washes in 2×SSC/0.1% SDS at room temperature for 15 minutes were followed by one wash in 1×SSC/0.1% SDS at 65°C for 15 minutes, two washes in 0.1×SSC/0.1% SDS at 65°C for 10 minutes, two washes in 0.1×SSC/0.1% SDS at 70°C for 10 minutes, two washes in 0.1×SSC/0.1% SDS at 78°C for 10 minutes, and two washes in 0.1×SSC/0.1% SDS at 85°C for 10 minutes. The blot was then exposed to film. For probe-making, the *ix* cDNA was cloned into the *Hin*cII and *Eco*RI sites of pBluescript KS II + (Stratagene, La Jolla, CA) as a *Msc*I-*Eco*RI fragment. The plasmid was linearized by digestion with *Cla*I, then transcribed by T7 RNA polymerase in the presence of [α-^{32}P]UTP.

As a loading control, the same blot was subsequently hybridized with a probe from the *ninaE* gene, which encodes the major rhodopsin, RH1 (O'Tousa et al., 1985). The *ninaE* gene was chosen as a control because the transcript is not expressed sex-specifically (data not shown). The *rp49* gene (O'Connell and Rosbash, 1984), typically used as a loading control, was found to be expressed at higher levels in females than in males (data not shown) and therefore was determined not to be a good loading control for comparing the two sexes. The *ninaE* probe was labeled with [α-^{32}P]dCTP by extension of random hexamers, using as a template a PCR-amplified region of the gene from the beginning of exon 2 through the beginning of exon 5 (nucleotide positions 365 through 1716 in GenBank Accession Number K02315). Hybridization conditions were as above for the *ix* probe. Two washes in 2×SSC/0.1% SDS at room temperature for 15 minutes were followed by one wash in 1×SSC/0.1% SDS at 42°C for 15 minutes, two washes in 0.1×SSC/0.1% SDS at 68°C for 15 minutes, and two washes in 0.1×SSC/0.1% SDS at 75°C for 15 minutes. Relative intensities of male and female signals for the *ix* hybridization and the *ninaE* hybridization were determined by analyzing scanned autoradiographs with NIH Image 1.62 software.

P-element-mediated germline transformation

To determine which one of the genes in the region to which *ix* had been localized was *ix*, two genomic rescue constructs were made and tested for their ability to rescue the *ix* phenotype. The 2GB construct was made by subcloning the 5.8-kb *Bam*HI-*Eco*RI genomic fragment from phage 2G into the CaSpeR4 vector (Pirrotta, 1988). The 1GS

construct was made by subcloning the 12-kb *Sal*I fragment from phage 1G into the *Xho*I site of the CaSpeR4 vector.

A knockout construct for each gene present in the 2GB construct, designated R, G and H, was generated to test for the inability to rescue the *ix* phenotype. The knockout construct, 3GBRΔ, which deletes the R gene after amino acid (aa) 16, was derived from the 2GB construct by the following procedure. The 2GB DNA was digested with *Spe*I and *Eco*RI, the ends were filled in with the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA), and the 9.7 kb *Spe*I-*Eco*RI CaSpeR4 vector + genomic DNA fragment was isolated. In a separate reaction the 2GB plasmid was digested with *Spe*I, the ends were filled in with Klenow, and the 1.7-kb *Spe*I genomic DNA fragment was isolated. The 1.7-kb *Spe*I blunt-ended DNA fragment was ligated to the 9.7-kb *Spe*I-*Eco*RI blunt-ended DNA fragment. To identify the desired construct, the DNA from candidate clones was digested with *Pst*I to determine the orientation of the 1.7 kb *Spe*I fragment and to confirm the presence of the 0.7 kb deletion. The knockout constructs for the G and H genes, 3GBG* and 3GBH* respectively, were derived from the 3GB construct, which contains a 1.0 kb deletion that removes the adjacent *tRNA:SeC* and *trypsin iota* genes by the following procedure. The 3GB construct is a derivative of the 2GB construct. The 2GB DNA was digested with *Bgl*II and *Eco*RI, the ends were filled in with Klenow, and then the DNA was ligated to itself generating a 1.0 kb deletion. For the 3GBG* construct, a stop codon was inserted at amino acid 91 by digesting the 3GB plasmid with *Sst*II, recessing the ends with T4 DNA polymerase (New England Biolabs, Beverly, MA), and ligating a 12 bp linker, NheI* (New England Biolabs, Beverly, MA), containing an *Nhe*I site and stop codons in all three reading frames, to the blunt-ended 3GB DNA. Candidate clones were screened for the presence of the unique *Nhe*I site and the absence of the unique *Sst*II site. The 3GBH* construct was made using the same steps as those used to make the 3GBG* construct, except the unique *Sfi*I site was used instead of the *Sst*II site and the stop codon was inserted at aa 44. Both 3GBG* and 3GBH* knockout constructs were further verified by DNA sequencing using the primers: G*1 (5′-CTCGCGGACAACTTAAAGAG) and H*1 (5′-GACAAGTTTTACGTGGAC).

Heat-shock-inducible cDNA (hscDNA) constructs were made to test rescue of the *ix* phenotype. The G and H cDNAs were subcloned into the *Hpa*I and *Not*I sites of the CaSpeRhs vector. cDNA H was inserted as a *Pvu*II-*Not*I fragment, and cDNA G2 was inserted as a *Hinc*II-*Not*I fragment into the same vector.

The 2GB, 1GS, and hscDNA constructs (0.3 μg/μl) were injected separately into w^{1118} embryos with the transposase source Δ2-3 (0.1 μg/μl) (Laski et al., 1986), following standard techniques (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The knockout constructs were injected at the concentration 0.4 μg/μl with the transposase source Δ2-3 (0.1 μg/μl) following the same method. G0 adults were crossed to w^{1118}*; Sp/CyO; Sb/TM2* flies of the opposite sex to identify transformants. All F_1 progeny with pigmented eyes were crossed to w^{1118}*; Sp/CyO; Sb/TM2* flies of the opposite sex to determine into which chromosome the construct inserted. The transgenes inserted into either the X or third chromosome were tested for rescue of the ix^2 mutation. Larvae carrying the hscDNA constructs were grown at 29°C continuously or heat shocked at 37°C for 1 hour each day during larval growth to assay rescue of the ix^2 mutation.

DNA sequencing

The genomic sequence of gene G was determined by sequencing the RΔ construct genomic DNA from gene H to gene R on both strands by cycle sequencing using dye termination reactions (Applied Biosystems, Foster City, CA). The primers used in the sequencing reactions were:

G#1, 5′-GAAAACAATTCGCGGCTGTTCAATATTTT;
G#4, 5′-TGCGCGGCACTAATCAGAGTGTCGTGT;
G#7, 5′-TTCACCCTGGAAATGTTGTCCAATTTTTCGGCCT;
G#8, 5′-CAAGGACTACCCAATATTTCATATTGTTACATACAT-AAAAGT;
G#S1, 5′-CTCGCGGACAACTTAAAGAG;
G#S2, 5′-TCACACGCATGCACTTAAGTTAAG;
R#S2, 5′-CTTCATTGCAGGTGGGTG; and
5′UTR#2, 5′-ATGAGATGACAGCTCTTTCCGGTCGGTTGAC-ATTAGCTA.

RNase protection assay

Total RNA from wild-type (Oregon-R) females and males was isolated from 4- to 5-day-old adult flies using TRIzol reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions. mRNA was purified from total RNA by binding of polyA+ RNA to $dC_{10}T_{30}$ oligonucleotides linked to polystyrene-latex beads (Qiagen, Valencia, CA). For probe-making, the 457 bp *Msc*I-*Pst*I genomic DNA fragment containing the *ix* translation initiation codon was subcloned into the *Hinc*II and *Pst*I sites of pBluescript KS II + (Stratagene, La Jolla, CA). The plasmid was linearized by digestion with *Xho*I, then transcribed by T7 RNA polymerase in the presence of [α-^{32}P]UTP, producing a uniformly labeled 527-bp antisense *ix* probe. The full-length probe was excised from a 5% acrylamide (19:1 acrylamide:N,N′-methylenebisacrylamide)/8 M urea gel and eluted in 350 μl of 0.5 M ammonium acetate/1 mM EDTA/0.2% SDS for 2 hours at 37°C. The RNase protection assay was performed using reagents supplied by Ambion (Austin, TX) according to the manufacturer's instructions. PolyA+ female and male RNA (1.8 μg) were each combined with 15 μl of the probe eluate. Two control tubes containing 50 μg of total yeast RNA and 15 μl of probe eluate were also prepared. Sample and probe were allowed to hybridize 16 hours at 42°C. The female and male fly RNA hybridization and one of the yeast control hybridization reactions were then digested with a 1:100 dilution of RNase mix (250 U/ml RNase A, 10,000 U/ml RNase T1); one yeast control hybridization was left undigested. The RNases were then inactivated and the nucleic acids precipitated and resuspended in 10 μl of gel loading buffer. The protected fragments were resolved on a 5% acrylamide/8 M urea gel. Only 10% of the yeast control without RNase digestion was loaded. ϕX174 DNA, digested with *Hae*III and labeled with [α-^{32}P]dATP by fill-in with T4 DNA polymerase, was also loaded as a size marker. After electrophoresing, the gel was dried on Whatman 3MM chromatography paper and autoradiographed.

5′ RACE and RT-PCR

PolyA+ RNA (200 ng per reaction) from w^{1118} females and males was reverse transcribed using gene-specific primer ix4 (5′-TGCGCG-GCACTAATCAGAGTGTCGTGT). The 5′ RACE system (Life Technologies, Rockville, MD) was used to amplify the 5′-end sequence of the *ix* transcript, by first adding an oligo-dC tail to the 3′ end of the cDNA with terminal transferase, then performing PCR with gene-specific primer ix12 (5′-CGATGGCGAGGATTGCATTA-CCTGCATCAT) and an anchor primer complementary to the oligo-dC, followed by nested PCR amplification with gene-specific primer ixL (5′-GGCATCATGTTCATGTTGGGATTCAT) and a second anchor primer. The PCR conditions were 94°C for 1 minute; 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes; then 72°C for 7 minutes. These amplification products were cloned and sequenced. A corresponding RT-PCR experiment using the same PCR conditions was performed on the same first-strand cDNA reactions (without dC-tailing) using a gene-specific primer, 5′UTR3 (5′-AATGCTAAATGAAACATTACACATCGTTTTTATTTGGGA), instead of the RACE anchor primers, for the two nested amplification reactions. RT-PCR products appearing to be splice variants because of their smaller size than predicted from genomic sequence, were cloned and sequenced.

3′ RACE

Wild-type (Canton-S) total RNA (5 μg per reaction) from females and

males was reverse transcribed using an oligo-dT-containing adapter primer (Life Technologies, Rockville, MD). This first-strand cDNA was then subjected to PCR amplification: 94°C for 1 minute; 30 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute; then 72°C for 7 minutes, using gene-specific primer ix943U (5′-TTAAAGAGGGACACGGGTGC) and a universal amplification primer with sequence matching the non-oligo-dT segment of the adapter primer (Life Technologies, Rockville, MD). A second, nested PCR amplification reaction was performed using gene-specific primer ix1032U (5′-CTTGAAGACGGCGATGCAGT) and the same universal amplification primer. These amplifications yielded single products of approximately 350 bp for both female and male RNA. The amplification products were cloned and sequenced.

CPRG assay

The *lacZ* activities were measured according to a previously published protocol (Coschigano and Wensink, 1993) incorporating published modifications (Li and Baker, 1998b).

Statistical analysis

The Yp data were analyzed using a two-factor analysis variance (ANOVA), with *ix* genotype and transgene presence/absence as fixed main effects for the pML-58 experiments and with *ix* genotype and *dsx* genotype as fixed main effects for the pCR1 experiments. To detect interactions between *ix* and *dsx,* the pCR1 experiment data were log-transformed before ANOVA, as multiplicative effects in the raw data become additive in the transformed data. Bristle counts for vaginal teeth, LTRB and sixth sternite were found to have heterogeneous variances among genotype classes, so the non-parametric Mann-Whitney U test (1-tailed) was used to detect differences between the classes. A G-test with the Yates correction was used to analyze the dorsolateral anal plate data. For sixth-tergite pigmentation, arcsin-transformed data were analyzed by one-tailed Student's *t*-test.

Yeast two-hybrid assays

The Matchmaker Gal4 two-hybrid system (BD Biosciences Clontech, Palo Alto, CA) was used according to the manufacturer's protocols. Briefly, full-length IX-, DSX^F- and DSX^M-coding sequences were cloned into the pAD and pBK vectors, which were co-transformed in pairs into the AH109 yeast strain and plated on -Ade, -His, -Leu and -Trp restrictive medium. Transformants that grew on this restrictive medium were further assayed for positive interactions by a colony-lift β-galactosidase assay.

Cell culture and co-immunoprecipitation

IX, DSX^F and DSX^M coding sequences were cloned in frame into the pAc5.1/V5-HisB or pMT5.1/V5-His (Invitrogen Corporation, Carlsbad, CA) vectors. To generate AU1-tagged constructs, the V5 epitope and polyhistidine regions of the V5-tagged constructs were replaced by digestion with *Bst*BI and *Pme*I and ligation with an oligonucleotide dimer (5′-GTT/CGAAGACACCTATCGCTATA-TACGTA/CCGGTCA) containing an in-frame AU1 epitope (Covance, Princeton, NJ). *Drosophila* S2 cells were cultured in Schneider's *Drosophila* medium (Gibco) in 10% FCS. Transfections were performed using Effectene reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol and as described elsewhere (Mosher and Crews, 1999). Briefly, cells were washed in PBS pH 7.4 and plated in media at a density of $5\text{-}7\times10^5$ cells/ml in a six-well plate (1.6 ml/well). Plasmid (~2 μg) and Effectene mixture was added and cells were grown for 24 hours prior to induction with 500 mM copper sulfate for an additional 24 hours.

Cells were washed in PBS and nuclear extracts (200 μl/well) were prepared as described elsewhere (Huang and Prystowsky, 1996). Extracts were normalized to equal protein concentrations and 100 μl samples were incubated with 2 μl monoclonal anti-AU1 antibody (Covance, Princeton, NJ) for 1 hour at room temperature. Bovine serum albumin was added to 2% final volume and lysates were incubated with Protein G Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) for an additional 2 hours at room temperature. Beads were pelleted, washed and transferred to SDS loading buffer. Proteins were resolved on a 12% SDS gel and probed via western blot using rabbit polyclonal anti-V5 antisera (Medical & Biological Laboratories, Nagoya, Japan) according to standard protocols.

Electrophoretic-mobility shift assay

Drosophila S2 cells were cultured and transfected as described above. Nuclear extracts (200 μl/well) were prepared as described above. Probe fragments were made by ^{32}P end-labeling the 185 bp *Cla*I-*Bgl*II FBE fragment of the Yp promoter region described previously (Burtis et al., 1991). Extracts (10 μl) were incubated in binding buffer [4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.05 mg/ml poly-dI-dC, protease inhibtor cocktail (Roche Applied Science, Indianapolis, IN, catalog number 1697498)] and 2 μl (50-100K cpm) probe was added for 20 minutes at room temperature. Monoclonal anti-V5 (Invitrogen Corporation, Carlsbad, CA) or anti-AU1 (Covance, Princeton, NJ) antibody was added in samples for super-shift where indicated. Proteins were resolved via native PAGE (4% acrylamide, 5% glycerol, 0.5×TBE) and complexes were visualized via autoradiography.

RESULTS

Cytological and physical localization of *intersex*

Complementation tests with deficiencies and loss-of-function alleles of *ix* localized *ix* to the cytological region 47E-47F11-18 (Chase and Baker, 1995). In addition to the previously characterized deficiencies, two new deficiencies were tested (Fig. 2A). *Df(2R)17* fails to complement *ix* and *Df(2R)27* complements *ix*. These complementation results place *ix* in the cytological region 47F between the *Df(2R)27* breakpoint at 47F1 and the $Df(2R)ix^{i3}$ breakpoint at 47F11-18. However, one complementation test with $Df(2R)ix^{87i3}$ gave a result that was not consistent with that localization of *ix*. $Df(2R)ix^{87i3}$ complemented a temperature-sensitive allele, ix^4, at the nonpermissive temperature, although it failed to complement all other *ix* alleles tested (Chase and Baker, 1995). After *ix* was molecularly identified, it was determined that the $Df(2R)ix^{87i3}$ chromosome contains a more complex rearrangement. In addition to the deletion of 47D-47F11-18, at least 6 kb of DNA containing *ix* (which is located approximately 70 kb from the 47F11-18 deletion breakpoint) was transposed to cytological position 50 on chromosome arm 2R. Thus, the complementation of the temperature-sensitive allele of *ix* by this deficiency chromosome was due to the transposed *ix* locus. A chromosomal walk was completed through the 47F interval and the relevant deficiency breakpoints were mapped to the DNA in the walk (Fig. 2B). The region between the *Df(2R)27* and $Df(2R)ix^{87i3}$ breakpoints, within which *ix* is located, is 100 kb. To localize *ix* in this 100 kb region, restriction fragment length polymorphism (RFLP) mapping was carried out (see Materials and Methods). This narrowed the region of interest to 65 kb.

Identification and characterization of *intersex* candidate genes

To locate candidate genes in the 65 kb region identified by the RFLP mapping, phage clones covering the entire region were used as probes to isolate cDNAs (B. C. W., C. M. G.-E., E. Williams and M. L. Goldberg, unpublished). Five cDNA classes

were identified (Fig. 3A). However, for most of the classes only one cDNA was isolated, raising the possibility that other genes may reside in this region and were not detected. Analysis of the *Drosophila* genome sequence (Adams et al., 2000) indicated that four additional transcripts (CT25954, CT25948, CT32444 and CT25938) are predicted in this region. Some or all of these predicted transcripts represent potential additional candidate genes. In addition, a *tRNA:SeC* gene and a cluster of trypsin genes (Wang et al., 1999) were previously mapped to the *ix* region.

P-element-mediated germline transformation experiments were undertaken to determine whether one of the genes identified in the 65 kb region was *ix*. The genomic rescue construct 2GB, which contains a 6 kb segment from the proximal part of the 65 kb *ix* walk, encompassing three genes of unknown function, as well as the *tRNA:SeC* and *trypsin iota* genes, was tested first for rescue of the *ix* phenotype (Fig. 3B). Two 2GB lines with insertions on the third chromosome were tested for rescue of the ix^2 mutation, and one line with the transgene inserted on the second chromosome was recombined onto the *Df(2R)en*^{B} chromosome and then tested for rescue of the ix^2/*Df(2R)en*^{B} phenotype. The somatic phenotype of the *ix* mutant females carrying the transgenes ranged from fully rescued (normal female development) to no rescue of the intersexual phenotype, depending on the lines tested and whether one or two copies of the transgenes were present. Because the *ix* phenotype was rescued for some of the *ix* mutant females, *ix* is one of the genes contained within the 2GB construct (Fig. 3B). The variable rescue of the *ix* phenotype suggested that this 6 kb genomic construct containing the *ix* gene was sensitive to position effect. Transformants with an overlapping construct 1GB were tested, and this construct also rescued the ix^2/*Df(2R)en*^{B} phenotype (Fig. 3B). The region of overlap between these two constructs is 4.5 kb and contained the three candidate genes R (CG12384), G (CG13201) and H (CG12352), but not the *tRNA:SeC* and *trypsin iota* genes.

To ascertain whether the DNA sequence of these three genes might indicate which is most likely *ix,* a cDNA representing each gene was sequenced. The amino acid sequence of each predicted protein was compared with sequences in the GenBank CDS translation, PDB, SwissProt, PIR and PRF databases using the PSI Blast program. Gene R encodes a protein that is 43% identical and 52% similar to the human death associated protein 1 (DAP1) (Deiss et al., 1995). The predicted H protein is 19% identical and 37% similar to the *Saccharomyces cerevisiae* ARD-1 (arrest-defective-1) protein (Whiteway and Szostak, 1985), and 22% identical and 43% similar to a N-acetyltransferase ARD-1 human homolog. Gene G encodes a novel protein. The sequence similarities of the candidate genes did not indicate that one gene was a better *ix* candidate than the others.

P-element-mediated germline transformation experiments were carried out with additional genomic constructs, to determine which candidate gene is *ix*. The approach taken was to knock out each candidate gene individually, while leaving the other two genes intact and to assay each of these derivatives of 2GB for the inability to rescue the *ix* phenotype. The

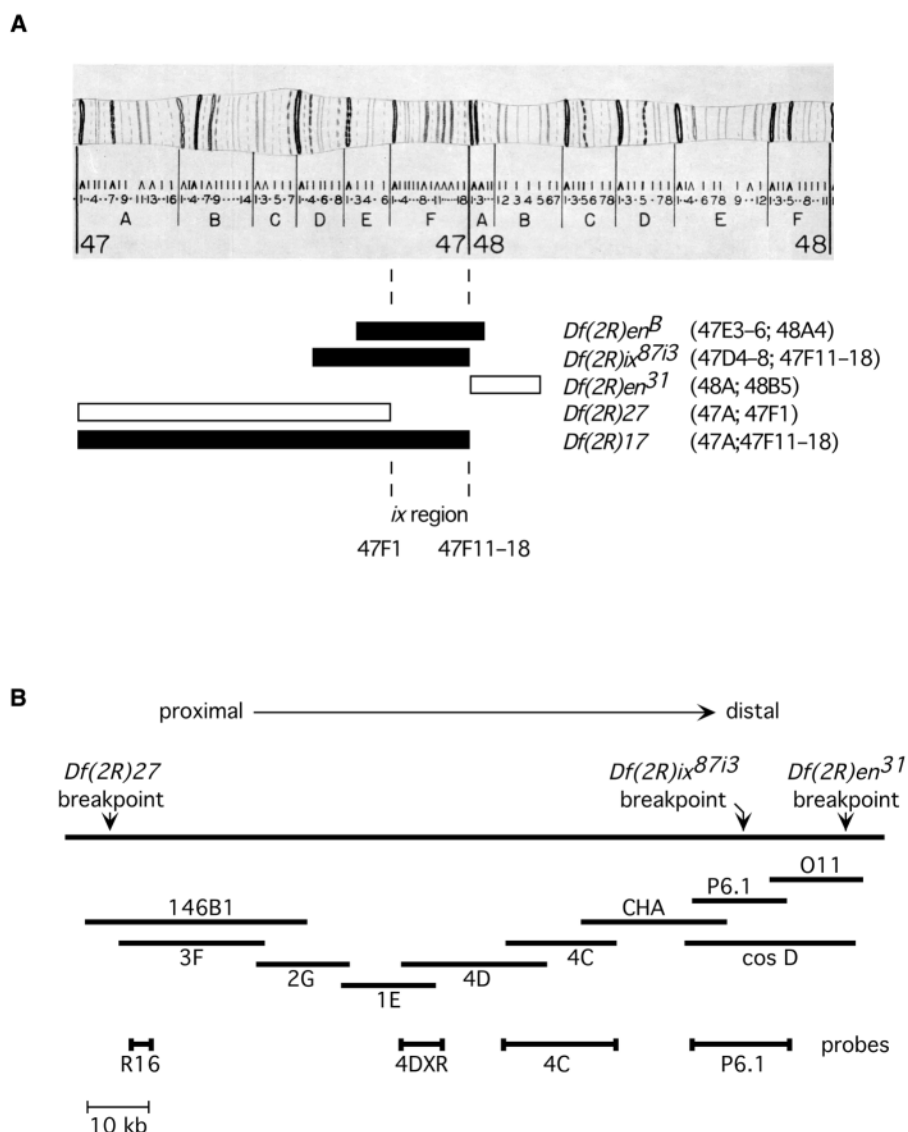

Fig. 2. The cytological and physical localization of *ix*. (A) Deficiency mapping of *ix*. The boxes indicate the region of the chromosome deleted for each deficiency chromosome (reported breakpoints in parentheses after deficiency names). The black boxes represent the deficiencies that fail to complement *ix*, and the white boxes represent the deficiencies that complement the loss-of-function alleles of *ix*. (B) Chromosomal walk spanning *ix*. Cosmid and phage clones spanning the cytological region 47F are indicated by lines. The relevant deficiency breakpoints are indicated above the DNA walk, and the probes used for RFLP mapping are indicated below the DNA clones. Map in A, reproduced (with permission) from Bridges and Bridges (Bridges and Bridges, 1939).

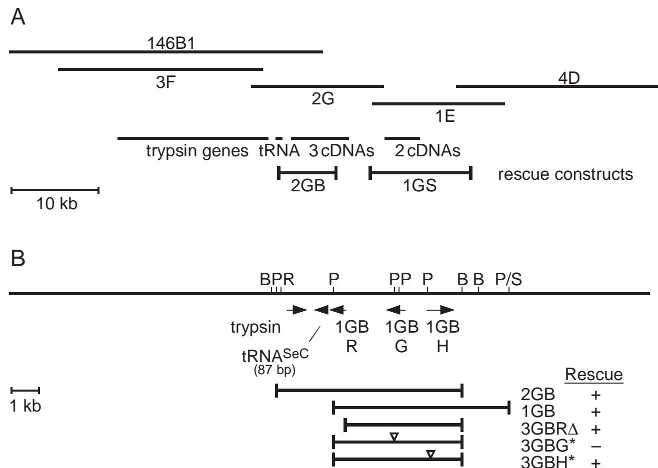

Fig. 3. The *ix* region defined by RFLP mapping and *P*-element-mediated germline transformation. (A) 65 kb *ix* region. cDNAs and known genes are indicated below the phage and cosmid clones in the *ix* region, and the genomic rescue constructs 2GB and 1GS are shown below the cDNAs. (B) Restriction-site map of the *ix* region defined by the 2GB genomic rescue construct. Transcripts included in the 2GB construct are indicated as arrows. The extents of germline transformation constructs are shown below the map, with rescue results indicated. Triangles indicate the positions of inserted stop codons. B, *Bam*HI; P, *Pst*I; R, *Eco*RI; S, *Sal*I.

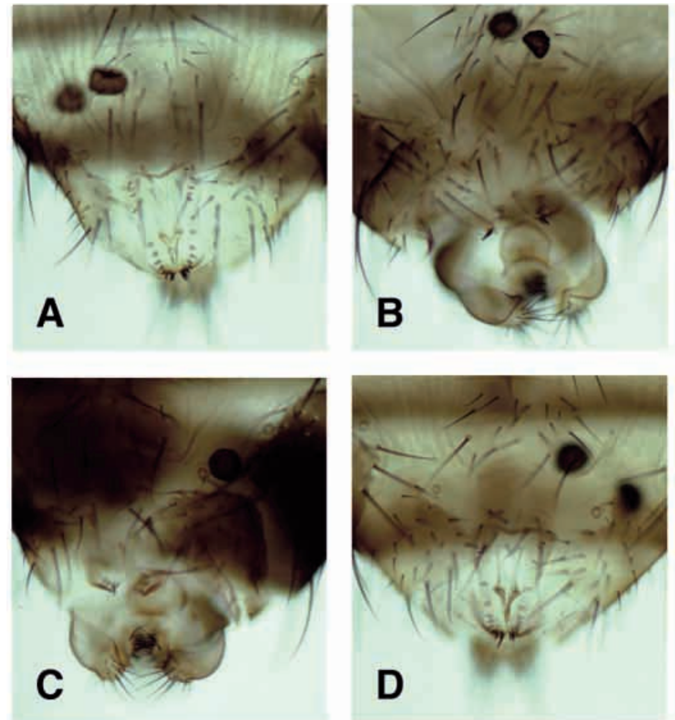

Fig. 4. Cuticle preps of wild-type and *ix*-mutant females. (A) Abdomen of wild-type female. (B) Abdomen of ix^2/ix^2 female. (C) Abdomen of ix^2/ix^2 female carrying one copy of the 3GBG* transgene. (D) Abdomen of ix^2/ix^2 mutant female carrying two copies of the heat-shock-inducible G cDNA transgene.

3GBRΔ construct deletes all but the first 16 amino acids of the R protein, the 3GBG* construct introduces a stop codon in the middle of the G protein at amino acid position 91, and the 3GBH* construct introduces a stop at amino acid position 44 of the H protein. For the 3GBRΔ construct, 21 lines were isolated with 13 insertions on the third chromosome, for the 3GBH* construct 43 lines were isolated with 18 insertions on the third chromosome, and for the 3GBG* construct 21 lines were isolated with 13 insertions on the third chromosome. The transgenic lines with insertions on the third chromosome were tested for rescue of the ix^2 phenotype. All 13 of the 3GBG* lines failed to rescue the *ix* phenotype (Fig. 3B, Fig. 4). For comparison, only one out of the 13 3GBRΔ lines and 1 of the 10 3GBH* lines tested failed to rescue the *ix* phenotype. This analysis of the knockout transgenes indicated that candidate G was *ix*.

To establish unequivocally that candidate G was *ix*, a heat-shock-inducible cDNA construct for gene G was tested for rescue of the *ix* phenotype. One of the five lines tested for the hscDNA G construct partially rescued the ix^2 phenotype when the larvae were grown continuously at 29°C (Fig. 4). Females carrying two copies of the transgene were rescued for the somatic defects but were not fertile, and females with one copy of the transgene were partially rescued. The results of experiments with the 3GBG* and hscDNA G transgenes showed that gene G is *ix.*

intersex sequence analysis

The *ix* gene encodes a protein of 188 amino acids (Fig. 5A,B). Analysis of the GenBank, EMBL and DDBJ EST databases using the Gapped Blast program identified mammalian ESTs and predicted proteins with significant similarity to the *ix* protein. The functions of the genes represented by these ESTs are unknown. From amino acids 15 to the C terminus of IX, the longest EST, a mouse EST (AA388092), is 37% identical and 52% similar to the *ix* protein; this mouse EST does not show similarity to the N-terminal 15 amino acids of IX. This similarity is highest in a 35 amino acid region of these proteins from amino acid 95 to amino acid 129. The sequence in the 35 amino acid region is 55% identical and 74% similar between the *ix* protein and either the mouse EST or a very similar human EST (U46237) (Fig. 5D). The stop codon introduced in the 3GBG* rescue construct is located just before this region. If the truncated G* protein is stable, the 35 amino acid region or a region after it must be required for *ix* function. Additionally, from amino acid 20 to the C terminus of IX, two predicted human proteins (XP_046121 and DKFZp434H247.1) are 35% identical and 50% similar to IX.

Comparison with sequences in the GenBank CDS translation, PDB, SwissProt, PIR and PRF databases using the PSI Blast program with aa position 3 to 47 in the N-terminal region of the *ix* protein revealed sequence similarity to the human synovial sarcoma translocation (SYT) protein (Clark et al., 1994), mouse SYT protein (de Bruijn et al., 1996) and the *C. elegans suppressor of ras* protein (SUR-2) (Singh and Han, 1995). In the 44 amino acid region of similarity, the *ix* protein is 45% identical and 51% similar to the human SYT protein, 50% identical and 52% similar to the mouse SYT protein, and 42% identical and 47% similar to SUR-2 (Fig. 5C).

The *sur-2* gene was identified as a suppressor of the *ras* multivulva phenotype (Singh and Han, 1995). Genetic epistasis analysis placed *sur-2* at the same position as transcription

factors in the vulval signal transduction pathway (Singh and Han, 1995), suggesting that the *sur-2* protein may function as a transcription factor.

The SYT protein is proposed to act as a transcriptional activator (Brett et al., 1997). In vitro analysis of SYT indicates that the 155 amino acid region of SYT with the highest transcriptional activation function contains the 44 amino acid sequence with similarity to *ix* (Brett et al., 1997). The sequence similarity of the IX protein to a region of the SYT protein that is capable of activating transcription raises the possibility that *ix* may function as a transcriptional activator.

Regulation of *intersex* by the sex-determination hierarchy

Because the *ix* phenotype is female specific and some genes in the somatic sex-determination hierarchy are regulated at the level of splicing, it was conceivable that the *ix* pre-mRNA would be sex-specifically spliced. However, no introns were identified by comparing the genomic sequence with the *ix* cDNA sequence, and Northern analysis did not detect sex-specific transcripts (Fig. 6A). In both males and females, a single hybridizing RNA species of approximately 750 bp was observed, consistent with the expected transcript size as determined by 5′ and 3′ RACE, which is 734-766 bases (start position 612-626, end position 1332, tail 28-46 bases, Fig. 5A). The start position determined by 5′ RACE is variable in both males and females but does not show a sex-specific difference. The relative signal intensity in males and females for the *ix* northern hybridization was normalized by the relative signal intensity in males and females for hybridization to *ninaE*. The *ix* transcript is ~8.7 times as abundant in wild-type females as in wild-type males. Preliminary data (not shown) from *ix* mutant germline clones in females, and from RNA analysis of females lacking a germline, suggest that the difference in transcript levels between females and males may be due to high *ix* expression in ovaries. The northern hybridization, cDNA analysis and 5′ RACE results suggest that the *ix* transcript is not sex specific and is not spliced.

However, sequence analysis of the genomic region just upstream of the transcription start site of the *ix* gene identified by 5′ RACE revealed a potential exon and intron. The putative exon would encode 33 amino acids and contain a consensus donor splice site (Fig. 5A). RT-PCR experiments, using a 5′ PCR primer that begins upstream of and extends into the putative exon, detected products that were of the size expected from the genomic DNA and smaller, apparently spliced, products were sometimes observed (data not shown). The RT-PCR result raises the possibilities that the transcription start determined by 5′ RACE is not correct or that a transcript initiating from an upstream start site is also expressed but at a much lower level, and was not detected by northern analysis or in the cDNAs isolated.

To confirm the *ix* pre-mRNA was not sex-specifically processed, RNase protection assays of polyA+ RNA isolated from males and females were performed using a probe that could distinguish between the spliced and unspliced products (Fig. 6B). RNase protection assays depend on neither reverse transcription nor amplification of the RNA as RT-PCR does, and RNase protection assays are more sensitive than northern analysis and could detect a rare transcript. The major protected

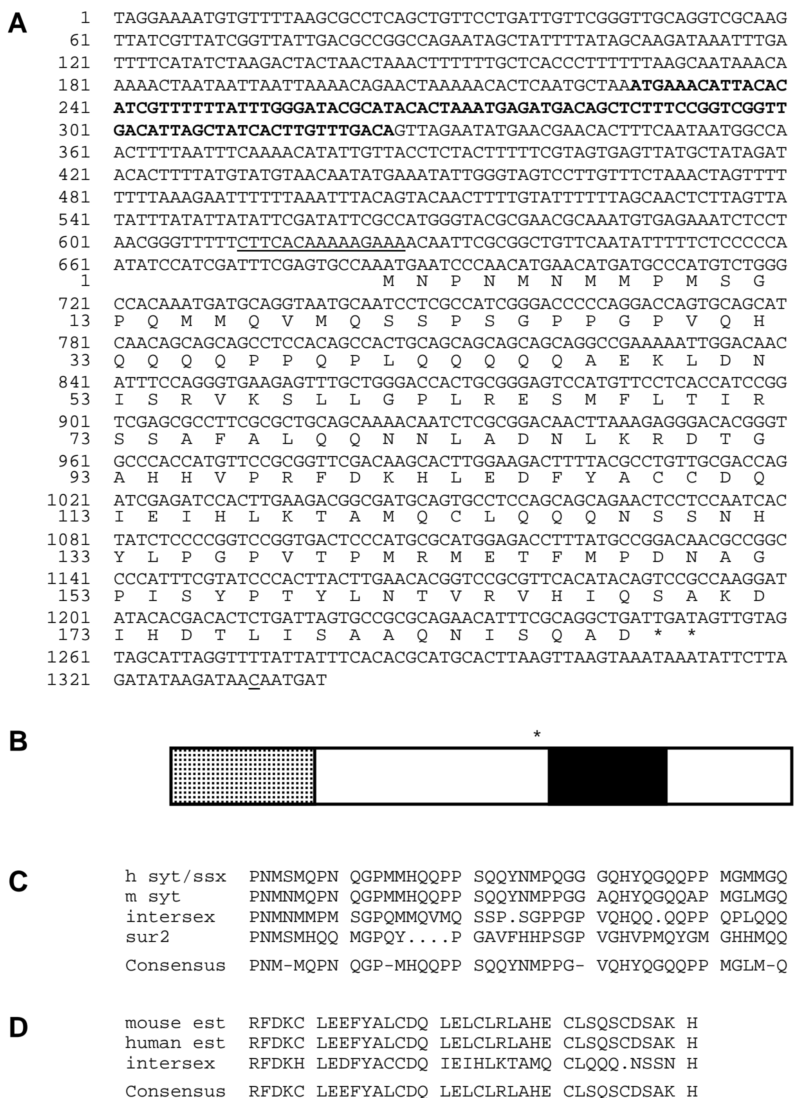

Fig. 5. *ix* DNA sequence and predicted protein product. (A) DNA and protein sequences. The *ix* DNA sequence (GenBank Accession Number, AF491289) is shown with the predicted protein sequence in single-letter code below the corresponding nucleotides. 5′ RACE experiments defined the start of the 5′ UTR (underlined), and 3′ RACE experiments determined the 3′ end of the mRNA (position at which polyadenylation begins underlined). The putative upstream exon suggested by results of RT-PCR experiments is indicated in bold. (B) Schematic of the predicted *ix* protein. The asterisk indicates the stop codon inserted in the 3GBG* knockout construct. The gray and black boxes represent regions of the *ix* protein with sequence similarity to known proteins and ESTs. (C) Sequence alignment of the N-terminal region of the *ix* protein (gray in B) with the mammalian SYT and *C. elegans sur-2* proteins. The consensus sequence is shown below. (D) Sequence alignment of a region of the *ix* protein (black in B) with the predicted proteins of human and mouse EST sequences. The consensus sequence is shown below.

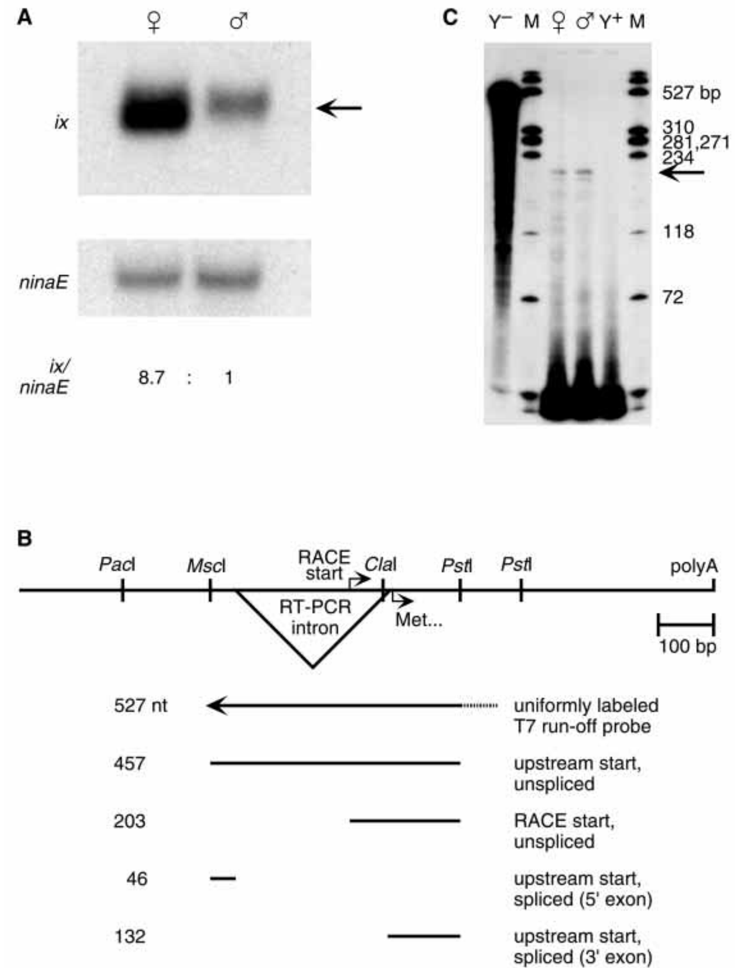

Fig. 6. Regulation of *ix* transcription. (A) Northern hybridization of female and male polyA+ RNA with probes from *ix* and *ninaE*. The arrow points to the position to which a 750 nucleotide molecule would migrate, as determined by a size marker run on the gel that was blotted (not shown). The relative abundance of female and male *ix* transcripts is given at the bottom, normalized to the amounts of *ninaE* transcript in each lane. (B) Scheme for RNase protection assay. A restriction map of the genomic region surrounding the putative *ix* translation start site (indicated by arrow labeled 'Met…') is shown, with the locations indicated of the putative transcription start site (as identified by 5′ RACE, labeled 'RACE start'), of the polyadenylation signal sequence (as identified by 3′ RACE, labeled 'polyA'), and of the potential intron from a transcript originating 5′ to the RACE start (as identified by RT-PCR analysis, labeled 'RT-PCR intron'). Aligned below the map is the full-length, 527 nucleotide probe used for the assay, which stretches from the *Msc*I site to the 5′-most *Pst*I site, and includes sequences from the T7-promoter-containing vector used to produce it (dashed region of arrow). Below the probe are the predicted protected fragments corresponding to the different potential *ix* transcripts. An unspliced transcript originating 5′ to the *Msc*I site would protect a probe fragment of 457 nucleotides, whereas an unspliced transcript originating at the site identified by 5′ RACE would protect a probe fragment of 203 nucleotides. If a transcript originating 5′ to the *Msc*I site were spliced at the donor and acceptor sites identified by RT-PCR, this processed transcript would protect two probe fragments, 46 nucleotides and 132 nucleotides in length. (C) RNase protection assay. Female and male polyA+ RNA samples were each hybridized in solution with the probe shown in B, then digested with RNase and electrophoresed. Yeast RNA controls were also performed, either with ('Y+') or without ('Y–') RNase. Size markers are in lanes marked 'M' and the sizes of marker bands are indicated at right. The arrow points to the position to which a 203 nucleotide molecule would migrate.

fragment is approximately 200 bp (Fig. 6C), as expected for an unspliced transcript that begins at the site indicated by 5′ RACE. Additionally, no qualitative difference between male and female protected fragments was observed. These results agree with the northern data, cDNA analysis and 5′ RACE results, and indicate the *ix* pre-mRNA is not spliced. Therefore, alternative processing of the *ix* transcript is not responsible for the female-specific *ix* phenotype, suggesting that *ix* functions together with one or more female-specific proteins to achieve the sex-specificity of the *ix* phenotype.

ix regulation of terminal differentiation

As *ix* functions at approximately the same position in the sex determination hierarchy as *dsx,* we carried out genetic experiments to ascertain whether *ix* cooperates with, or functions independently of, *dsx* to control female sexual differentiation. We examined how *ix* and *dsx* function relative to one another in controlling Yp gene expression and the development of an array of sexually dimorphic cuticular structures.

We first focused on the role of *ix* in controlling Yp gene expression. Previous studies identified the Fat Body Enhancer (FBE) in the *Yp1* and *Yp2* intergenic region as necessary and sufficient for the sex-specific expression of both *Yp1* and *Yp2* (Garabedian et al., 1986). DSX regulates Yp gene expression through three DSX binding sites in the FBE (An and Wensink, 1995; Burtis et al., 1991; Coschigano and Wensink, 1993) and northern analysis suggests that ix^+ was required for DSX^F mediated activation of *Yp1* transcription (Waterbury et al., 1999). To confirm that *ix* regulates Yp gene expression and to determine whether *ix* activates Yp expression through the same regulatory region as *dsx,* the expression of Yp reporter gene constructs was assayed in wild-type and *ix* mutant females.

Our analysis of the expression levels of the pML-58 Yp reporter construct (provided by M. Lossky and P. Wensink), which contains the FBE and 196 bp of the *Yp1* and *Yp2* intergenic region fused to the *lacZ* gene, indicates that this region is sufficient for *ix* regulation of the Yp genes in females. Chromosomal females either homozygous or heterozygous for an *ix* mutation and either carrying or not carrying an ix^+ transgene were compared. Including the transgene in the analysis allows definitive assignment of an effect on reporter expression to *ix* and not to a linked locus. A 1.9-3.5-fold reduction in *lacZ* activity from pML-58 reporter-construct expression was observed comparing homozygous and heterozygous *ix*-mutant females (Fig. 7A, ANOVA genotype main effect $P<0.0001$). Therefore there is a significant effect of the *ix* genotype on expression of the Yp reporter construct regardless of the presence or absence of the *ix* transgene. Additionally, the effect of the *ix* transgene on expression of the Yp gene reporter construct is to increase *lacZ* activity (ANOVA transgene main effect $P<0.0001$). There is no significant interaction between the *ix* genotype and the presence of the *ix*

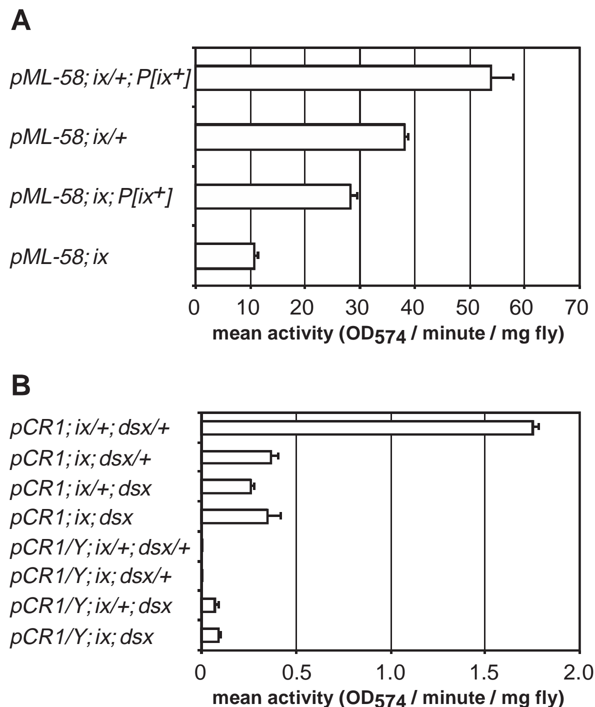

Fig. 7. *ix* and *dsx* act interdependently to activate Yp reporter expression in females. (A) Progeny from *pML-58/pML-58; ix^3/SM; ry/ry* mothers crossed to *w/Y; ix^2/CyO; P[ix$^+$ 9.5]/MKRS* fathers. (B) Progeny from *pCR1/pCR1; ix^3/CyO; dsx p^p/MKRS* mothers crossed to *w/Y; Df(2R)en^B/CyO; dsx^{127}/MKRS* fathers. Mean *lacZ* activity is plotted for each progeny genotype, in units of OD_{574}/minute/mg fly, based on the CPRG assay. Error bars are +1 s.e.m. Each genotype was assayed at least in triplicate.

transgene (ANOVA interaction P=0.74), indicating that adding one wild-type copy of the *ix* gene, either at the *ix* locus or via the transgene, increases Yp reporter gene expression equivalently. As the *ix*-mutant females assayed are heteroallelic (*ix^2/ix^3*) and one copy of the *ix* transgene rescues the decreased Yp reporter construct expression observed in these *ix*-mutant females, the reduction in Yp gene expression is due to the *ix* mutation and not another mutation on the second chromosome. These results indicate that the *ix* protein activates transcription of the Yp gene reporter construct in females through a region that contains the DSX^F DNA-binding sites, raising the possibility that IX interacts with DSX^F to regulate expression of the Yp genes.

To investigate whether *ix* regulation of the Yp genes is dependent on *dsx* activity, expression of the pCR1 Yp reporter construct (Lossky and Wensink, 1995), which contains the entire *Yp1* and *Yp2* intergenic region fused to a *lacZ* reporter gene, was analyzed in *ix*, *dsx* and *ix; dsx* double mutant flies. The use of the full intergenic region, including the *her* responsive region (HRR) outside the FBE (Li and Baker, 1998b), increases the resolution of the analysis, as *her* upregulates Yp expression approximately fivefold, except when DSX^M is present, thereby amplifying expression differences due to the presence or absence of DSX^M activity. pCR1 expression was reduced in both *ix* mutant females (Fig. 7B, ANOVA *ix* genotype main effect P=0.0001) and *dsx* mutant females (ANOVA *dsx* genotype main effect P<0.0001). If *ix* and *dsx* act independently to regulate Yp expression, then the combined effect of the *ix; dsx* mutant would be the product of the individual mutant effects. Log-transforming the data makes multiplicative effects additive, so the *ix*×*dsx* interaction term in the ANOVA is an indicator of the independence of the effects of the two loci. The interaction between *ix* and *dsx* is highly significant (P<0.0001), indicating a strong dependent relationship between the two loci. In males, the *ix* genotype has no effect on the level of pCR1 expression (Fig. 7B, P=0.4), but the *dsx* genotype has a significant effect on pCR1 expression (P<0.0001). The interaction between *ix* and *dsx* is not significant (P=0.71). These results indicate that *ix* does not function in males to regulate Yp gene expression. Therefore, the *ix* protein only functions in females and cooperates with DSX^F to activate Yp gene expression.

In addition to regulating Yp expression, DSX^F controls the development of sexually dimorphic cuticular structures (Baker and Ridge, 1980; Li and Baker, 1998b). Because the *ix* phenotype is indistinguishable from the *dsx* female phenotype, IX may also interact with DSX^F to regulate these aspects of female differentiation. However, *her,* another gene with a phenotype similar to *ix* and *dsx,* cooperates with *dsx* to control female differentiation of foreleg bristles and tergites 5 and 6, but functions independently of *dsx* to regulate development of vaginal teeth and anal plates in females (Li and Baker, 1998b). If *ix* acts independently of *dsx* to regulate some aspect of terminal sexual differentiation, then in *ix; dsx* mutant females that aspect of sexual differentiation would be masculinized compared with the individual mutants. However, if the genes function together then the phenotype of *ix; dsx* double mutants would be the same as that of the single mutants. To test these possibilities, the phenotypes of five sexually dimorphic cuticular structures in *ix*, *dsx* and *ix; dsx* mutant flies were assayed.

The first cuticular phenotype examined was the number of vaginal teeth in females. There are on average 26.6 vaginal teeth on *ix/+; dsx/+* females (Table 1, row 1) and 0 vaginal teeth on *ix/+; dsx/+* males (Table 1, row 5). The intersexual *ix* and *dsx* single mutant females had on average 9.7 vaginal teeth and 6.0 vaginal teeth, respectively, significantly fewer than wild-type females [Table 1, compare rows 1 and 2 (P<0.0001), and rows 1 and 3 (P<0.0001)]. The *ix; dsx* mutant females formed on average 6.45 vaginal teeth, indicating that loss of wild type *ix* function does not masculinize *dsx* mutant females (Table 1, rows 3 and 4, P=0.77). This result indicates that *dsx* is dependent on wild-type *ix* activity for vaginal teeth development in females. Elimination of wild-type *dsx* function appears to weakly masculinize the *ix* mutant females (Table 1, rows 2 and 4, P=0.032). However, this effect may be due to the fact that the EMS-induced *ix^3* allele is a strong loss-of-function allele but is not completely null (Chase and Baker, 1995). The nucleotide sequence of *ix^3* is consistent with this inference, in that the only difference between *ix^3* and the *ix$^+$* allele of its progenitor stock is a T to A substitution in nucleotide 1221, which results in a Ser to Arg amino acid substitution (data not shown). We thus conclude that *ix* and *dsx* act interdependently to regulate differentiation of vaginal teeth in females.

The next sexually dimorphic structure analyzed was the anal plates. Females have one dorsal and one ventral anal plate, and males have two lateral anal plates. Intersexual flies have a pair

Table 1. *ix* and *dsx* act interdependently to promote female differentiation of sex-specific cuticular structures

Row*	Genotypes†	VT‡ Mean	VT‡ s.d.	DLAP§ %	T6¶ Mean	T6¶ s.d.	LTRB** Mean	LTRB** s.d.	S6†† Mean	S6†† s.d.
1	*XX; ix/+; dsx/+*	26.60	1.70	100	0.53	0.14	5.28	0.51	20.85	1.23
2	*XX; ix; dsx/+*	9.70	5.29	90	0.92	0.06	6.88	0.65	21.10	1.68
3	*XX; ix/+; dsx*	6.00	3.55	65	0.96	0.05	7.75	0.78	19.50	1.91
4	*XX; ix; dsx*	6.45	2.11	85	0.95	0.04	7.25	0.54	20.75	1.83
5	*XY; ix/+; dsx/+*	0.00	0.00	0	0.97	0.03	10.60	1.06	0.30	0.66
6	*XY; ix; dsx/+*	0.00	0.00	0	0.96	0.03	9.65	0.86	0.45	0.69
7	*XY; ix/+; dsx*	6.00	4.19	70	0.95	0.04	7.45	0.60	17.35	2.06
8	*XY; ix; dsx*	6.85	2.74	80	0.96	0.03	7.25	0.54	18.20	1.58

*Rows 1-8 are results from siblings and can be compared.

†The flies analyzed are progeny of *pCR1/pCR1; ix*^{3}*/CyO; dsx p*^{p}*/MKRS* mothers crossed to *w/Y; Df(2R)en*^{B}*/CyO; dsx*^{127}*/MKRS* fathers.

‡Vaginal teeth numbers.

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

¶The percentage width of the sixth tergite that is darkly pigmented.

**The numbers of the last transverse row of bristles on the foreleg basitarsus. In wild-type males, it is the bristle number of a sex comb.

††The numbers of bristles on the sixth sternite.

of dorsolateral plates that are often fused at the dorsoanterior side. When collecting the data for all genotypes, the wild-type female dorsal anal plate was considered fused. All *ix/+; dsx/+* females had fused dorsal anal plates (DLAP) compared with 65% of the *dsx* mutant females [Table 1, compare rows 1 and 3 (P=0.0073)] and 90% of the *ix* mutant females [Table 1, compare rows 1 and 3 (P=0.46)] that had fused DLAP. Although 90% of the *ix* mutant females had fused DLAP, these anal plates appeared intersexual because the two anal plates were not completely fused into one anal plate. Thus the absence of statistical significance should not be taken as evidence against a sex-transforming effect of *ix* on the anal plates of females. Similar to the single mutant phenotypes, 85% of the *ix; dsx* mutants had fused DLAP [Table 1, compare rows 3 and 4 (P=0.27) and rows 2 and 4 (P=1)], indicating that the *ix; dsx* double mutant phenotype is not stronger than the single mutant phenotypes. Therefore, *ix* and *dsx* do not act independently in the anal-plate precursor cells to control female-specific differentiation of anal plates.

Two other sexually dimorphic cuticular phenotypes analyzed were the extent of pigmentation of the sixth tergite and the development of the last transverse row of bristles (LTRB) on the basitarsus, which form the sex combs in males. With respect to pigmentation of tergite 6, the *ix/+; dsx/+* females had on average 53% pigmentation (Table 1, row 1) and the *ix/+; dsx/+* males had 97% pigmentation (Table 1, row 5). The loss of wild-type *ix* or *dsx* activity increased pigmentation of the sixth tergite significantly, to 92% and 96%, respectively [Table 1, compare rows 1 and 2 (P<0.0001), and rows 1 and 3 (P<0.0001)]. Similarly, in the *ix; dsx* double mutant females, the extent of pigmentation was 95% [Table 1, compare rows 3 and 4 (P=0.65), and rows 2 and 4 (P=0.045)]. Therefore, the double mutant phenotype is comparable with the single mutant phenotypes. The results for the analysis of differentiation of LTRB were the same as for pigmentation of tergite 6. In *ix/+; dsx/+* females, an average of 5.28 bristles formed compared with 10.6 bristles on *ix/+; dsx/+* males (Table 1, rows 1 and 5). The number of bristles increased significantly, to 6.8 in *ix* mutant females and 7.75 in *dsx* mutant females [Table 1, compare rows 1 and 2 (P<0.0001), and rows 1 and 3 (P<0.0001)], and the *ix; dsx* double mutant females had 7.25 bristles, similar to the single mutants [Table 1, compare rows 3 and 4 (P=1.0) and rows 2 and 4 (P=0.004)]. For both sixth-tergite pigmentation and LTRB differentiation, the *ix; dsx* mutant females were weakly masculinized compared with the *ix* mutant females but not the *dsx* mutant females. Again, this observation is presumably due to the fact that the *ix*^{3} allele is not completely null and residual *ix* activity is eliminated in the double mutant by loss of *dsx* function. These results indicate that for female-specific development of the LTRB and pigmentation of the sixth tergite, *ix* and *dsx* function cooperatively.

The last cuticular phenotype examined was the number of bristles formed on the sixth sternite. Females have an average of 20.85 bristles (Table 1, row 1) and males have an average of 0.30 bristles (Table 1, row 5) on the sixth sternite. Previous analysis indicated that bristles form on the sixth sternite of females independent of the activity of *dsx* and *her* (Li and Baker, 1998b). A comparison of the phenotypes of *ix* mutant females and *ix/+; dsx/+* females demonstrates that the development of these bristles is also independent of *ix* function [Table 1, compare rows 1 and 2 (P=0.73)]. In this analysis the *dsx* mutant females had a slight reduction in the number bristles, from 20.85 to 19.50 [Table 1, compare rows 1 and 3 (P=0.0045)]. However, this weak effect of the *dsx* genotype was not observed in previous studies and may represent differences in the genetic background of these flies.

The analysis of these sexually dimorphic cuticular structures in males indicated that *ix* does not function in males. Unlike the *dsx* mutant males, *ix* mutant males did not develop vaginal teeth nor do they have fused dorsal lateral anal plates (Table 1, rows 6 and 7). The extent of pigmentation of the sixth tergite and differentiation of the LTRB on the basitarsus were also unaffected (Table 1, rows 5 and 6). The wild-type activity of both *dsx* and *her* is required in males to prevent bristle formation on the sixth sternite (Li and Baker, 1998b). However, loss of *ix* function did not increase bristle formation on S6 [Table 1, compare rows 5 and 6 (P=0.18)]. These results confirm previous results that *ix* only functions in females (Chase and Baker, 1995).

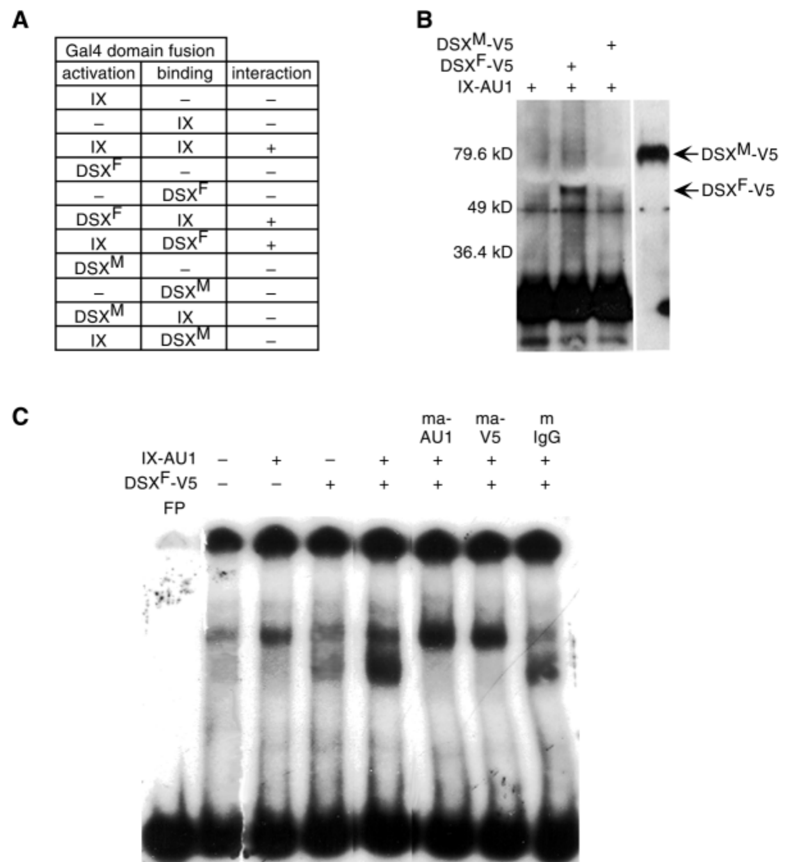

Fig. 8. Interaction of IX and DSX^{F} to form a DNA-binding complex. (A) Results from yeast two-hybrid analysis. Fusion constructs between IX, DSX^{F} or DSX^{M} protein-coding sequences and either the Gal4 activation domain coding sequence (column 1) or the Gal4 DNA-binding domain coding sequence (column 2) were co-transformed into yeast containing Ade, His and *lacZ* reporters. A minus sign in column 1 or 2 indicates that no construct bearing the given domain sequence was transformed. The presence of an interaction between the fusion proteins (plus sign in column 3) was inferred by the ability of a transformed strain to grow on restrictive medium and to express *lacZ*, using a colony lift assay. (B) Co-immunoprecipitation of DSX^{F} and IX. Nuclear extracts containing equivalent concentrations of protein from *Drosophila* S2 cells co-transfected with AU1-epitope-tagged IX and V5-epitope-tagged DSX^{F} (lane 2) or DSX^{M} (lane 3) were immunoprecipitated with monoclonal anti-AU1 antibody and analyzed via western blot with rabbit polyclonal anti-V5. The band at approximately 57 kDa in lane 2 corresponds to DsxF-V5. Lane 4 contains the supernatant from extracts precipitated in lane 3, indicating that DsxM-V5 protein was expressed but not precipitated with Ix-AU1. (C) DSX^{F} and IX form a DNA-binding complex. EMSA was performed by incubating nuclear extracts from S2 cells expressing DSX^{F}-V5 and/or IX-AU1 with a ^{32}P-labeled DNA probe containing the 185 bp FBE region of the Yp enhancer, and resolving by native PAGE. Lane 1, free probe (no extract); lanes 2-5, probe plus tagged protein (indicated above lanes). Extracts from cells co-transfected with DSX^{F}-V5 and IX-AU1 were probed in the presence of anti-AU1 or anti-V5 monoclonal antibodies or mouse IgG (lanes 6-8, respectively) to assay super-shifting of the DNA-binding complex.

In all cases, we examined in which *ix* and *dsx* regulate female differentiation of cuticular structures – formation of vaginal teeth, development of the dorsal anal plate, development of the LTRB and pigmentation of the sixth tergite – the phenotype of the *ix; dsx* double mutant females was not masculinized compared with the *ix* and *dsx* single mutant phenotypes. Although the analysis of vaginal teeth differentiation, pigmentation of the sixth tergite, and formation of LTRB revealed that elimination of wild type *dsx* activity weakly masculinized *ix* mutant females, this effect probably represents the elimination of residual *ix* activity because the *ix* alleles may not be complete loss of function alleles (Chase and Baker, 1995). Therefore, the results of the phenotypic analysis of sexually dimorphic cuticular structures and the Yp gene reporter constructs indicate that *ix* and *dsx* act interdependently to regulate all aspects of female terminal differentiation.

Physical interaction of IX and DSX^{F}

Given the sex-specific effect of *ix* mutants, and the dependence of DSX^{F} function on *ix* genotype, we sought to determine if IX interacts directly with DSX^{F} to regulate transcriptional targets such as the Yp genes. As a preliminary test for such an interaction, we used a yeast 2-hybrid assay to look for an interaction between IX and either the DSX^{F} or DSX^{M} proteins. Constructs fusing full-length IX, DSX^{F} or DSX^{M} proteins with the Gal4 activation or DNA-binding domains were created and co-transformed into a yeast strain containing metabolic and enzymatic reporters for Gal4 function. Positive interactions were assayed by growth on restrictive medium as well as by *lacZ* expression (see Materials and Methods). By these criteria, the IX fusion proteins exhibit positive homomeric interaction, and exhibit heteromeric interaction with DSX^{F}, but not DSX^{M}, fusion proteins (Fig. 8A). Control transformants, containing only single constructs, failed to demonstrate either growth on restrictive medium or *lacZ* expression.

To confirm the results of our two-hybrid assay, we performed co-immunoprecipitation of tagged IX and DSX proteins expressed in *Drosophila* S2 cells. Constructs capable of expressing IX tagged with an AU1 epitope and either DSX^{F} or DSX^{M} tagged with a V5 epitope were co-transfected into S2 cells, extracts of which were subsequently immunoprecipitated using monoclonal anti-AU1 antibody. The immunoprecipitates and supernatants were resolved via SDS-PAGE and analyzed by western blot with polyclonal anti-V5. The AU1-epitope-tagged IX is able to co-immunoprecipitate DSX^{F}-V5 but not DSX^{M}-V5, indicating that IX specifically forms a stable complex with DSX^{F} in vivo (Fig. 8B). Analysis of supernatants confirmed that all proteins were expressed upon induction.

Because DSX^{F} functions as a transcription factor, we sought to determine if the complex between IX and DSX^{F} proteins is able to bind DNA effectively. We performed electrophoretic-mobility shift assay (EMSA) using as probe the previously characterized 185 bp FBE region of the Yp enhancer, which contains DSX-binding sites (Burtis et al., 1991). Nuclear extracts from S2 cells transfected with the epitope-tagged constructs discussed above were incubated with ^{32}P end-labeled FBE fragments and resolved by native PAGE. A stable DNA-binding complex was seen in extracts containing IX and DSX^{F} (Fig. 8C, lanes 1-5). To confirm that this complex

contained IX and DSX^F, extracts were incubated with probe in the presence of one of three antibodies – anti-AU1, anti-V5 or nonspecific mouse IgG. That the predominant DNA-binding complex is specifically super-shifted by antibodies to the individual tags indicates that the complex contains minimally IX and DSX^F (Fig. 8C, lanes 6-8).

DISCUSSION

To begin to understand how *ix* regulates the terminal differentiation genes in females and how the sex-specificity of the *ix* phenotype is achieved, we have cloned the *ix* gene. The N-terminal 44 amino acids of IX share sequence similarity with the human and mouse synovial sarcoma translocation (SYT) proteins (Clark et al., 1994; de Bruijn et al., 1996) and *C. elegans* SUR-2 (Singh and Han, 1995). The remainder of IX has sequence similarity to mammalian ESTs. The function of the genes represented by these ESTs is unknown.

The SYT and SUR-2 proteins are proposed to function as transcription factors (Brett et al., 1997; Singh and Han, 1995). Human SYT was first identified as a chimeric protein resulting from a chromosomal translocation that is implicated in synovial sarcomas (Clark et al., 1994), and the region of the SYT protein that activates transcription in in vitro assays (Brett et al., 1997) contains the region with similarity to the *ix* protein. The SYT chimeric protein is nuclear, as expected for a transcription factor (Brett et al., 1997; dos Santos et al., 1997). As SYT does not contain a known DNA-binding motif (Clark et al., 1994), it is thought to form a complex with a DNA-binding protein to activate transcription.

The sequence similarity of IX to the human and mouse SYT proteins and to SUR-2 suggests that *ix* may also act as a transcription activator. Additionally, like the SYT proteins, IX does not contain a recognizable DNA-binding domain. *dsx* and *her* function at the same position in the hierarchy as *ix* and these genes encode proteins with zinc-finger DNA-binding domains (Erdman and Burtis, 1993; Li and Baker, 1998a). However, neither DSX^F nor HER proteins can activate transcription alone in 2-hybrid assays (this paper; H. Li, data not shown), suggesting these proteins lack activation domains and interact with additional proteins to regulate the expression of the terminal differentiation genes in females. The DSX^M protein has a 152 amino acid male-specific C terminus, whereas the smaller DSX^F protein has only 30 unique amino acids at its end (Burtis and Baker, 1989). Therefore, the DSX^F protein may need to interact with a co-factor for female-specific activity. The genetic results in this paper, indicating that *dsx* and *ix* act interdependently to regulate female-specific differentiation, and the biochemical results, indicating the DSX^F and IX physically interact, suggest that IX may be this co-factor. It remains to be determined whether the specific interaction of DSX^F and IX is mediated through the 30 amino acid C terminus of DSX^F.

As the *ix* phenotype is female specific and expression of other genes in the somatic sex determination hierarchy is controlled sex-specifically, expression of the *ix* gene could have been sex-specifically regulated. However, XY flies expressing a cDNA corresponding to DSX^F are phenotypically female (Waterbury et al., 1999) instead of intersexual, suggesting that *ix* protein is present in these chromosomal males. Our analysis of *ix* cDNAs, northern hybridization and RNase protection assays demonstrated that the *ix* pre-mRNA is not sex-specifically spliced. Therefore, the female-specific phenotype is not achieved through alternative processing of the *ix* transcript. The previous genetic results and our molecular results suggest that the *ix* protein is present in both females and males and its female-specific function is mediated through interactions with the female-specific protein DSX^F.

Analysis of Yp gene expression demonstrated that *dsx*, *her* and *ix* control Yp gene expression in the fat body (An and Wensink, 1995; Burtis et al., 1991; Coschigano and Wensink, 1993; Li and Baker, 1998b; Waterbury et al., 1999). Our results indicate that *ix* acts through the Yp intergenic region that contains the DSX-binding sites. Additionally, expression of DSX^F in *ix* mutant males is not sufficient to activate Yp expression (Waterbury et al., 1999), suggesting DSX^F requires IX to regulate Yp expression. Our analysis of Yp reporter constructs in *ix; dsx* mutant females also suggests that IX and DSX^F act together to control Yp gene transcription. Therefore, DSX^F may require IX as a co-factor to directly regulate Yp gene expression in females. This possibility is supported by the observation that IX and DSX^F are present in a complex that binds the region of the Yp FBE that contains DSX-binding sites.

Phenotypic analysis of *ix; dsx* mutant females demonstrated that *ix* and *dsx* also cooperate to regulate female-specific differentiation of sexually dimorphic cuticular structures. The *ix* mutation failed to masculinize the *dsx* mutant females, indicating that *dsx* is dependent on *ix* activity in the precursor cells that differentiate into the vaginal teeth, dorsal anal plates, last transverse row of bristles on the basitarsus and sixth tergite pigment-producing cells. Additionally, the phenotypic analysis of *ix* mutant males confirmed that *ix* does not function in males. The possibility that *ix* also functions with *her* to control female-specific differentiation of some sexually dimorphic structures remains to be tested. The tight interdependence of DSX^F and IX suggests that the relationship between HER and IX is likely to be the same as that between HER and DSX in females.

Understanding of the role of the sex determination hierarchy in sex-specific differentiation has been substantially revised and enhanced by recent studies that have begun to illuminate how information from the sex determination hierarchy is integrated with information from other developmental hierarchies. In particular, it had been thought that *dsx* played a mainly permissive role in the development of the internal and external genitalia. These structures develop from the genital imaginal disc, which is composed of three primordia deriving from embryonic abdominal segments A8, A9 and A10. The classical view of the genital disc was that the A8-derived primordium differentiated into female genital structures in females and was repressed in males, whereas the A9-derived primordium differentiated into male genital structures in males and was repressed in females; the A10-derived primordium differentiates into anal structures appropriate to the sex of the individual. Thus, whereas the differentiation of the anal primordium requires an instructive cue from the sex hierarchy, the differentiation of the appropriate genital primordium was inferred to require only a permissive function of the sex hierarchy, with segmental identity determining the structures that ultimately developed.

This classical view was overturned by the finding that the 'repressed' genital primordium in each sex actually develops into adult structures: the 'repressed' female (A8) primordium produces a miniature eighth tergite in males and the 'repressed' male (A9) primordium produces the parovaria in females (Keisman et al., 2001). Consistent with its instructive role, the sex hierarchy actively modulates the regulation by other developmental pathways of sex-specifically deployed genes. The *dachshund* (*dac*) gene is differentially expressed in the male and female genital discs, and the sex hierarchy mediates this sex-specific deployment by determining cell-autonomously whether *dac* is activated by *wingless* signaling (in females) or by *decapentaplegic* signaling (in males) (Keisman and Baker, 2001). Fibroblast growth factor (FGF) signaling in the genital disc is also regulated cell-autonomously by the sex hierarchy (Ahmad and Baker, 2002). DSX^F represses the FGF-encoding *branchless* (*bnl*) gene, thus restricting *bnl*-expressing cells to the male genital disc. FGF signaling from these cells recruits into the disc mesodermal cells expressing the FGF receptor encoded by the *breathless* (*btl*) gene. Once inside the male genital disc, these *btl*-expressing cells become epithelial and eventually give rise to the paragonia and vas deferens, components of the internal male genitalia. An instructive role for the sex hierarchy is also evident in an adult tissue not derived from the genital imaginal disc. The *bric à brac* (*bab*) locus integrates signals from the homeotic genes, as well as the sex hierarchy to repress pigmentation of tergites 5 and 6 in females (Kopp et al., 2000).

Although the Yp genes, which are activated by DSX^F and repressed by DSX^M, are the only known direct target of *dsx*, it is likely that DSX^F acts in some cases to repress transcription and that DSX^M acts in some cases to activate transcription. Indeed, if the examples above represent cases of direct regulation, then it is clear that the effect of DSX^F or DSX^M is dependent upon both the cellular context and the promoter organization of the target gene. Such context-dependent duality of function finds precedent in several well characterized transcription factors. The mechanisms that determine whether a bi-functional transcription factor is in an activating or repressing state are diverse, and include binding of ligand co-factors, differential organization of binding sites in promoters, interaction with other DNA-binding factors, and concentration-dependent structural changes (Roberts and Green, 1995). The DSX proteins provide an especially interesting case of dual regulatory activity because not only are DSX^F and DSX^M each capable of activating some target genes and repressing others, but the two isoforms often have opposite effects, with DSX^F repressing those genes that DSX^M activates and vice versa. It may be that IX, functioning as a co-factor for DSX^F, plays a key role in effecting this symmetry of dual regulatory activities.

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REFERENCES

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

Ahmad, S. M. and Baker, B. S. (2002). Sex-specific deployment of FGF signaling in *Drosophila* recruits mesodermal cells into the male genital imaginal disc. *Cell* **109**, 651-661.

An, W. and Wensink, P. C. (1995). Integrating sex- and tissue-specific regulation within a single Drosophila enhancer. *Genes Dev.* **9**, 256-266.

Ashburner, M. (1989). Drosophila*: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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

Brett, D., Whitehouse, S., Antonson, P., Shipley, J., Cooper, C. and Goodwin, G. (1997). The SYT protein involved in the t(X;18) synovial sarcoma translocation is a transcriptional activator localised in nuclear bodies. *Hum. Mol. Genet.* **6**, 1559-1564.

Bridges, C. B. and Bridges, P. N. (1939). A new map of the second chromosome: a revised map of the right limb of the second chromosome of Drosophila Melanogaster. *J. Hered.* **30**, 475-476.

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

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

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

Clark, J., Rocques, P. J., Crew, A. J., Gill, S., Shipley, J., Chan, A. M., Gusterson, B. A. and Cooper, C. S. (1994). Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. *Nat. Genet.* **7**, 502-508.

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

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

de Bruijn, D. R., Baats, E., Zechner, U., de Leeuw, B., Balemans, M., Olde Weghuis, D., Hirning-Folz, U. and Geurts van Kessel, A. G. (1996). Isolation and characterization of the mouse homolog of SYT, a gene implicated in the development of human synovial sarcomas. *Oncogene* **13**, 643-648.

Deiss, L. P., Feinstein, E., Berissi, H., Cohen, O. and Kimchi, A. (1995). Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev.* **9**, 15-30.

dos Santos, N. R., de Bruijn, D. R., Balemans, M., Janssen, B., Gartner, F., Lopes, J. M., de Leeuw, B. and Geurts van Kessel, A. (1997). Nuclear localization of SYT, SSX and the synovial sarcoma-associated SYT-SSX fusion proteins. *Hum. Mol. Genet.* **6**, 1549-1558.

Erdman, S. E. and Burtis, K. C. (1993). The *Drosophila* doublex proteins share a novel zinc finger related DNA binding domain. *EMBO J.* **12**, 527-535.

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

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

Hays, T. S., Deuring, R., Robertson, B., Prout, M. and Fuller, M. T. (1989). Interacting proteins identified by genetic interactions: a missense mutation in α-tubulin fails to complement alleles of the testis-specific β-tubulin gene of *Drosophila melanogaster*. *Mol. Cell. Biol.* **9**, 875-884.

Huang, D. Y. and Prystowsky, M. B. (1996). Identification of an essential cis-element near the transcription start site for transcriptional activation of the proliferating cell nuclear antigen gene. *J. Biol. Chem.* **271**, 1218-1225.

Jallon, J.-M., Lauge, G., Orssaud, L. and Antony, C. (1988). Female pheromones in *Drosophila melanogaster* are controlled by the doublesex locus. *Genet. Res.* **51**, 17-22.

Keisman, E. L. and Baker, B. S. (2001). The *Drosophila* sex determination

hierarchy modulates *wingless* and *decapentaplegic* signaling to deploy *dachshund* sex-specifically in the genital imaginal disc. *Development* **128**, 1643-1656.

Keisman, E. L., Christiansen, A. E. and Baker, B. S. (2001). The sex determination gene *doublesex* regulates the A/P organizer to direct sex-specific patterns of growth in the *Drosophila* genital imaginal disc. *Dev. Cell* **1**, 215-225.

Kopp, A., Duncan, I. and Carroll, S. B. (2000). Genetic control and evolution of sexually dimorphic characters in *Drosophila*. *Nature* **408**, 553-559.

Laski, F. A., Rio, D. C. and Rubin, G. M. (1986). Tissue specificity of *Drosophila* P element transposition is regulated at the level of mRNA splicing. *Cell* **44**, 7-19.

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

Li, H. and Baker, B. S. (1998b). *hermaphrodite* and *doublesex* function both dependently and independently to control various aspects of sexual differentiation in *Drosophila*. *Development* **125**, 2641-2651.

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

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

Marín, I. and Baker, B. S. (1998). The evolutionary dynamics of sex determination. *Science* **281**, 1990-1994.

Mosher, J. and Crews, S. (1999). Effectene reagent yields high transfection efficiencies with *Drosophila melanogaster* S2 cells. *Qiagen News* **4**, 7-10.

Nagoshi, R. N., McKeown, M., Burtis, K. C., Belote, J. M. and Baker, B. S. (1988). The control of alternative splicing at genes regulating sexual differentiation in D. melanogaster. *Cell* **53**, 229-236.

O'Connell, P. and Rosbash, M. (1984). Sequence, structure, and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Res.* **12**, 5495-5513.

O'Tousa, J. E., Baehr, W., Martin, R. L., Hirsh, J., Pak, W. L. and Applebury, M. L. (1985). The Drosophila *ninaE* gene encodes an opsin. *Cell* **40**, 839-850.

Pirrotta, V. (1988). Vectors for P-mediated transformation in *Drosophila*. In *Vectors, a Survey of Molecular Cloning Vectors and their Uses* (ed. R. L. Rodriguez and D. T. Denhardt), pp. 437-456. Boston, MA: Butterworths.

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

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

Roberts, S. G. E. and Green, M. R. (1995). Dichotomous regulators. *Nature* **375**, 105-106.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Singh, N. and Han, M. (1995). *sur-2*, a novel gene, functions late in the *let-60 ras*-mediated signaling pathway during *Caenorhabditis elegans* vulval induction. *Genes Dev.* **9**, 2251-2265.

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341-347.

Stearns, T. and Botstein, D. (1988). Unlinked noncomplementation: isolation of new conditional-lethal mutations in each of the tubulin genes of *Saccharomyces cerevisiae*. *Genetics* **119**, 249-260.

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

Torok, T., Tick, G., Alvarado, M. and Kiss, I. (1993). P-lacW insertional mutagenesis on the second chromosome of *Drosophila melanogaster*: isolation of lethals with different overgrowth phenotypes. *Genetics* **135**, 71-80.

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

Wang, S., Magoulas, C. and Hickey, D. (1999). Concerted evolution within a trypsin gene cluster in *Drosophila*. *Mol. Biol. Evol.* **16**, 1117-1124.

Waterbury, J. A., Jackson, L. L. and Schedl, P. (1999). Analysis of the Doublesex female protein in *Drosophila melanogaster*. Role in sexual differentiation and behavior and dependence on Intersex. *Genetics* **152**, 1653-1667.

Whiteway, M. and Szostak, J. W. (1985). The ARD1 gene of yeast functions in the switch between the mitotic cell cycle and alternative developmental pathways. *Cell* **43**, 483-492.