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The role of the *ovarian tumor* locus in *Drosophila melanogaster* germ line sex determination

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SUMMARY

The locus *ovarian tumor* (*otu*) is involved in several aspects of oogenesis in *Drosophila melanogaster*. The possible role of *otu* in the determination of the sexual identity of germ cells has not been extensively explored. Some *otu* alleles produce a phenotype known as ovarian tumors: ovarioles are filled with numerous poorly differentiated germ cells. We show that these mutant germ cells have a morphology similar to primary spermatocytes and that they express male germ line-specific reporter genes. This indicates that they are engaged along the male pathway of germ line differentiation. Consistent with this conclusion, we found that the splicing of *Sex-lethal* (*Sxl*) pre-mRNAs occurs in the male-specific mode in otu^- transformed germ cells. The position of the *otu* locus in the regulatory cascade of germ line sex determination has been studied by using mutations that constitutively express the feminizing activity of the *Sxl* gene. The sexual transformation of the germ cells observed with several combinations of *otu* alleles can be reversed by constitutive expression of *Sxl*. This shows that *otu* acts upstream of *Sxl* in the process of germ line sex determination. Other phenotypes of *otu* mutations were not rescued by constitutive expression of *Sxl*, suggesting that several functions of *otu* are likely to be independent of sex determination. Finally, we show that the gene dosage of *otu* modifies the phenotype of ovaries heterozygous for the dominant alleles of *ovo*, another gene involved in germ line sex determination. One dose of otu^+ enhances the ovo^D ovarian phenotypes, while three doses partially suppress these phenotypes. Synergistic interaction between ovo^{D1} and *otu* alleles leads to the occasional transformation of chromosomally female germ cells into early spermatocytes. These interactions are similar to those observed between ovo^D and one allele of the *sans fille* (*snf*) locus. Altogether, our results imply that the *otu* locus acts, along with *ovo*, *snf*, and *Sxl*, in a pathway (or parallel pathways) required for proper sex determination of the female germ line.

Key words: sex determination, germ line, ovarian tumor, *Drosophila*, *Sex-lethal* splicing

INTRODUCTION

Sex determination of somatic cells in *Drosophila melanogaster* is regulated in a cell-autonomous manner by their chromosomal constitution (reviewed by Cline, 1988; Baker, 1989; Hodgkin, 1990; Steinmann-Zwicky et al., 1990). In a normal diploid cell having two sets of autosomes, a single X chromosome (1X:2A) leads to male development and two X chromosomes (2X:2A) lead to female differentiation. This X:A ratio functions early during development to initiate the expression of the *Sex-lethal* locus (*Sxl*) so that only female cells (2X:2A) contain functional Sxl proteins (Bopp et al., 1991; Keyes et al., 1992). The early difference between male and female somatic cells is then maintained by an autoregulatory mechanism in which the Sxl proteins control the splicing of their own pre-mRNAs. In the absence of Sxl proteins (male embryos), non-coding *Sxl* RNAs are produced, while in the presence of Sxl proteins (female embryos) alternative processing produces mRNAs encoding functional Sxl proteins (Bell et al., 1991; see Fig. 3A). Besides its own expression, *Sxl* controls two additional pathways in somatic cells: sexual differentiation via Sxl-dependent splicing of *tra* pre-mRNA and dosage compensation, which is the mechanism that hyperactivates the transcription of the single X chromosome in males to the level of the 2 X chromosomes in females (reviewed by Lucchesi and Manning, 1987).

In contrast to the single level of regulation observed in somatic cells, sex determination in the germ line does not depend solely on the chromosomal constitution of the germ cells, but is also influenced by the sex of the surrounding soma (Steinmann-Zwicky et al., 1989; Nöthiger et al., 1989;

reviewed in Pauli and Mahowald, 1990, Steinmann-Zwicky, 1992). 1X:2A germ cells develop as male independently of the sex of the surrounding somatic cells, although in a female soma they arrest early during spermatogenesis either because of the absence of a positive signal provided by the male soma or because of counteractive female somatic influence. In contrast, 2X:2A germ cells develop according to the sex of the surrounding soma, although in a male soma they do not differentiate further than early spermatocyte stage.

The nature of the somatic signal is not known, but one autosomal and three X-linked loci have been found to be required in female (2X:2A) germ cells for their proper sex determination (reviewed by Pauli and Mahowald, 1990). Mutations in these genes result in either of two different phenotypes: sex-specific germ cell death or transformation of the 2X:2A germ line toward male identity. The first phenotype is exemplified by recessive loss-of-function alleles of *ovo*. The germ cells of female embryos homozygous for null mutations of *ovo* die during embryogenesis (Oliver et al., 1987, 1990). This sex-specific germ cell death might be due to incorrect dosage compensation (see Discussion). The second phenotype, sometimes called ovarian tumor, is seen in mutations of *sans fille* (*snf*, Gollin and King, 1981; Oliver et al., 1988; Steinmann-Zwicky 1988; Salz 1992), in some alleles of *Sxl* (Schüpbach, 1985; Steinmann-Zwicky et al., 1989), in mutations of the *fl(2)d* locus (Granadino et al., 1992), in some combinations of *ovo* alleles, and from interaction of *ovo* and *snf* mutations (Oliver et al., 1990). Instead of normal egg chambers with one oocyte and fifteen nurse cells, ovarioles are filled with numerous undifferentiated germ cells resembling early spermatocytes. Similarly, male-like germ cells can be observed in the gonads of 2X:2A flies whose soma is phenotypically male because of mutations in somatic sex determination genes such as *tra* (Nöthiger et al., 1989). Transformation of the sexual identity of 2X:2A germ cells seems therefore to be a common consequence either of inappropriate somatic signaling or of mutations in several genes necessary within the female germ line.

Various alleles of the X-linked recessive female sterile locus *ovarian tumor* (*otu*) display either of the phenotypes described above, germ cell death and ovarian tumors, as well as other defects of oogenesis apparently unrelated to sex determination. Strong alleles such as otu^{10} have no, or few, female germ cells, which fail to differentiate. Several milder alleles like otu^{1} or otu^{13} produce primarily ovarian tumors. The weakest alleles have germ cells of female identity, which show a variety of defects during oogenesis such as abnormal number of nurse cells, polytene chromosomes in nurse cell nuclei, polyploidization of the oocyte nucleus, failure to transport the nurse cell cytoplasm into the oocyte at stage 11 of oogenesis and defective synthesis of glycogen-rich beta yolk spheres (King et al., 1986; Storto and King, 1987; 1988; Tirronen et al., 1992). Although *otu* has been cloned, the sequence of the two protein isoforms has not provided any information to account for the multiple functions suggested by the various phenotypes of *otu* mutations, except that early functions probably depend on the larger polypeptide (Steinhauer and Kalfayan, 1992).

In this paper, we focus on the ovarian tumors produced by *otu* mutations and show that the germ cells express genes normally active in the male but not in the female germ line. Molecular and epistatic analyses indicate that the *otu* locus is needed upstream of *Sxl* in the process of germ line sex determination. Furthermore, we show that *otu* alleles interact with the dominant female sterile mutations of *ovo*, in a manner similar to the snf^{1621} mutation. We therefore conclude that the *otu* locus has a critical function for the establishment of the sexual identity of the germ line in a pathway (or in parallel pathways) that includes the *ovo*, *snf*, *fl(2)d*, and *Sxl* genes.

MATERIALS AND METHODS

Genetics

Flies carrying the *Df(1)RA2* and *Df(1)KA14* deletions were obtained from the Indiana Stock Center (Bloomington, IN). Flies with the *Dp(1;2)FN107* duplication and the *otu* alleles were obtained from D. Mohler and are described in King et al. (1986), Sass et al. (1993) and Geyer et al. (1993). Full genotypes are given in Table 2. Stocks with lethal mutations mapping at 7F-8A were obtained from N. Perrimon and L. Engstrom and are described in Perrimon et al. (1989). Crosses were performed at 25°C.

Genetic interactions between a given mutation and the dominant female sterile alleles of *ovo* (ovo^{D}) were analyzed as described below. Briefly, virgin females were crossed to ovo^{D} males. The progeny were collected every day, aged for 7 days and females were dissected in PBS. Ovaries were squashed and observed under a compound microscope. For ovo^{D2} and ovo^{D3}, ovary development was quantified by counting the number of oocytes that had developed to stage 10 or later of oogenesis (see King, 1970, for stages). Flies doubly heterozygous for ovo^{D} and the mutation to be tested were compared to their siblings heterozygous for ovo^{D}. A non-parametric Smirnov statistical test (Connover, 1980) was used to analyze the data.

To test the effect of constitutive expression of *Sex-lethal*, recombinant chromosomes were generated between Sxl^{M1} or Sxl^{M4} and various *otu* alleles. The presence of the *Sxl* allele was tested by its ability to rescue female lethality at 25°C in the progeny of da^{1}/da^{1} females (Cline, 1978). The rescuing effect of constitutive expression of *Sxl* was analyzed in females of genotypes Sxl^{M1} otu^{x} / Sxl^{+} otu^{y}. Control flies (Sxl^{+} otu^{x} / Sxl^{+} otu^{y}) were also analyzed, and had phenotypes very similar to those described in detail by King et al. (1986) and Storto and King (1987, 1988)

Beta-galactosidase expression

The method of Gönczy et al. (1992) was slightly modified. Flies were aged for 8-12 days at 25°C before dissection in PBS or Ringer's buffer. Testes and ovaries were fixed for 15 minutes at room temperature in 1% glutaraldehyde in 50 mM sodium cacodylate pH 7.3. They were rinsed in Ringer's buffer once and then three times in staining buffer (10 mM NaH_2PO_4/Na_2HPO_4 pH 7.2, 1 mM $MgCl_2$, 150 mM NaCl, 5 mM $K_4[Fe^{II}(CN)_6]$ and 5 mM $K_3[Fe^{III}(CN)_6]$). Tissues were then placed in staining buffer at room temperature for at least 30 minutes. Just before use, 500 μl of staining buffer was prewarmed at 37°C and 10 μl of 10% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, dissolved in dimethylformamide and kept at −20°C) was added. Before addition to the tissues, the staining solution was centrifuged for 3 minutes to pellet the precipitate. Incubation was at 37°C for 16-36 hours.

Analysis of *Sxl* transcripts

$Poly(A)^{+}$ RNAs from whole flies, gonadectomized flies, or gonads were reverse transcribed with Moloney murine leukemia virus reverse transcriptase and oligo dT as primer (Sambrook et al.,

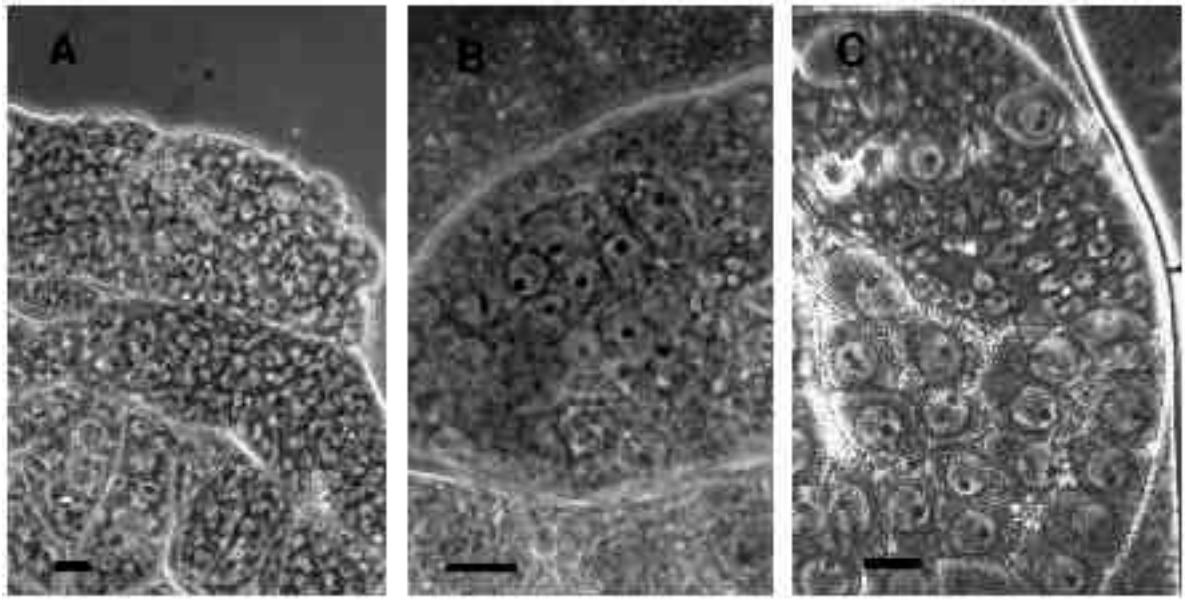

Fig. 1. Morphology of the germ cells in *otu* tumorous ovary. (A) Low magnification of egg chambers containing numerous 2X:2A germ cells which resemble small spermatocytes. Genotype: otu^1 / otu^1. (B) Higher magnification of these germ cells. (C) Tip of a testis. Genotype: *FM7a* / Y. Bars, 20 μm. Note that the size of the 2X:2A transformed germ cells rarely exceeds half the size of mature spermatocytes.

1989). These cDNAs were amplified by polymerase chain reaction (annealing at 54°C for the first 10 cycles and at 50°C for the next 20 cycles) using Taq DNA polymerase, in the presence of [^{32}P]dATP (Saiki et al., 1988). To detect only the male-specific mode of splicing, the following primers were used: non-sex-specific 5′ ACAACGACAGCAGCAGGCCA (position 286-305, Bell et al., 1988) and specific for the male exon 5′ GGGCTTTG-GAGGTGTCCTCG (starting 1 nucleotide upstream of the 3′ end of the male exon). DNA amplification products were analyzed on 5% sequencing gels. As expected (Bell et al., 1988; Samuels et al., 1991; B. Oliver, Y.-J. Kim and B.S. Baker, unpublished data), two amplification products of about 460 nucleotides were found. The identity of these products was confirmed by chain termination sequencing with Sequenase brand T7 DNA polymerase (USB) and the above primers, according to the manufacturer's instructions with the following exception. Template and primers were resuspended into sequencing buffer, boiled for 3 minutes and flash annealed in a dry ice/ethanol bath (Kusukawa et al., 1990).

RESULTS

Molecular evidence of sexual transformation of *otu*$^-$ germ cells

One of the striking phenotypes of flies homozygous or transheterozygous for combinations of *otu* mutations is the presence of egg chambers filled by poorly differentiated germ cells, which have been interpreted to be the result of uncontrolled cystocyte divisions (King et al., 1986). However, the resemblance of these germ cells to spermatocytes (large nucleus and prominent dark nucleolus whose center appears dark or light depending on the focal plane, see Fig 1) suggests the possibility that these germ cells are sexually transformed. We found that morphological criteria can be difficult to use. For instance the size of the cells and their nucleus will depend upon how far into spermatogenesis the cells have proceeded. We have therefore investigated the possible sexual transformation of 2X:2A ; otu^- (chromosomally female) germ cells using molecular markers.

Gönczy et al. (1992) have described two P[w^+ lacZ] enhancer traps (line 590 and line 606) that expressed the β-galactosidase reporter gene in the male germ line in both stem cells and mitotic phases of germ cell differentiation, but not in mature primary spermatocytes (Fig. 2A). Line 606 did not express the reporter gene in any other stages of male germ line differentiation or in the female germ cells. Line 590, which showed stronger enzyme activity, also accumulated β-galactosidase in mature sperm and in advanced vitellogenic stages (stage 10 and later). The expression of line 590 in the female germ line was extremely weak compared to that in the male germ line and late enough so that it did not interfere in the experiments described below.

The two P[w^+ lacZ] were introduced into several *otu* stocks chosen for their high proportion of apparently sexually transformed germ cells. Females homozygous for the P[w^+ lacZ] insert were dissected and their ovaries stained for β-galactosidase activity (Fig. 2). Except for the intensity of staining, similar results were obtained with line 590 and line 606. As expected, no expression was found in the phenotypically normal germ line of heterozygous females or in somatic follicle cells (Fig. 2B). In contrast, strong staining was observed in ovaries homozygous for otu^1, otu^3, otu^{11} or otu^{13}, or in heteroallelic combinations of these alleles (Fig. 2C). Staining was restricted to egg chambers containing morphologically transformed germ cells and was not found in egg chambers containing nurse cells (Fig. 2D). Variability in staining was dependent on the genotype: otu^1 homozygous ovaries showed a stronger β-galactosidase activity, possibly because abnormal egg chambers with this mutation are frequently larger and contain more cells than egg chambers produced by other *otu* alleles. There was also vari-

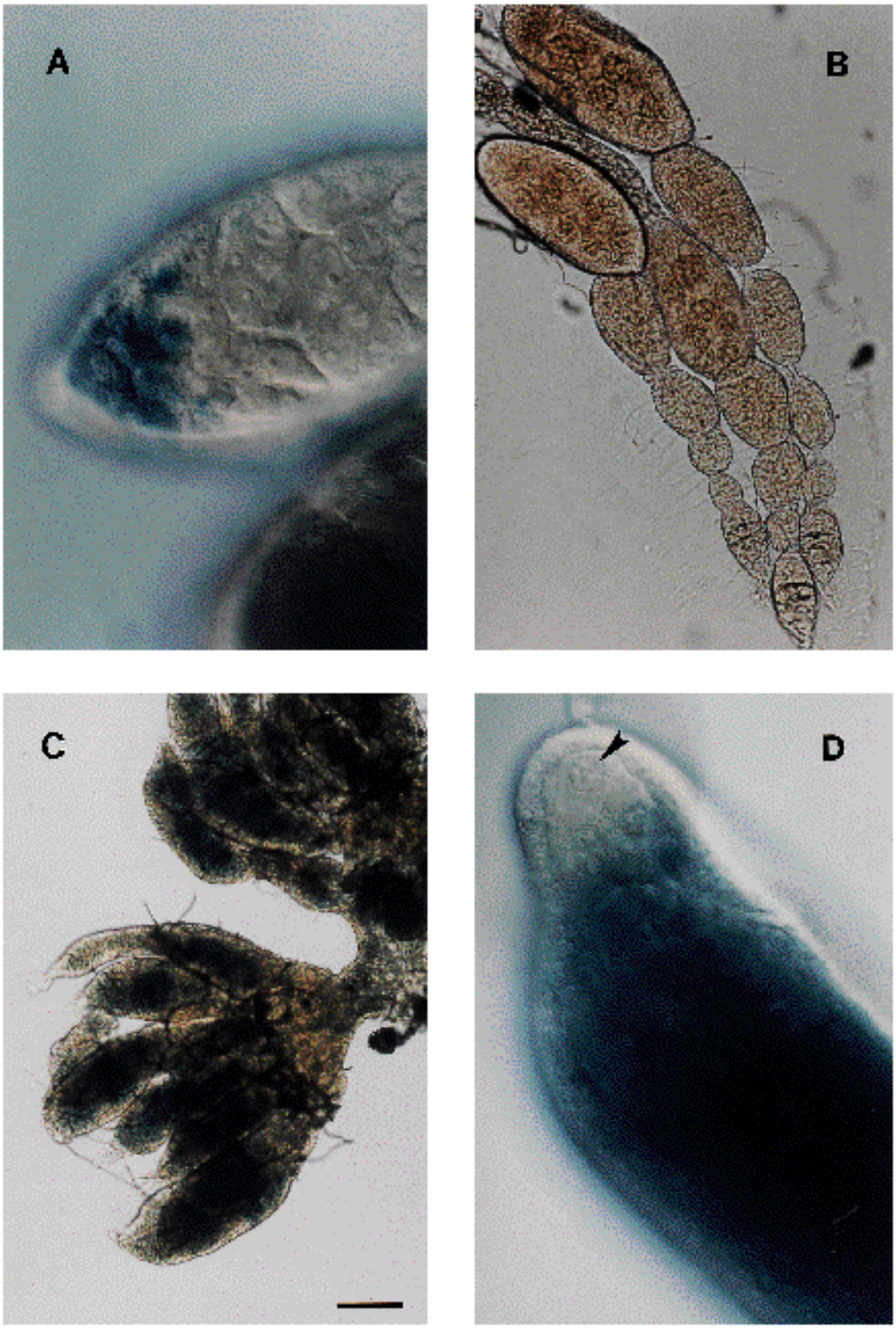

Fig. 2

ability between egg chambers and among cells within an egg chamber. This variability could indicate slightly different stages of differentiation along the male pathway and/or incomplete sexual transformation. Since 2X:2A ; *otu*⁻ germ cells are larger than spermatogonia, but not as large as mature spermatocytes (see Fig. 1), we conclude that they develop along the male pathway and that their differentiation is arrested at some point during the spermatocyte growth phase.

Alteration of the splicing of *Sex-lethal* transcripts by *otu* mutations

Genetic evidence indicates that in germ cells the *Sxl* gene acts downstream of both the somatic signal (Nöthiger et al., 1989; Steinmann-Zwicky et al., 1989) and the *snf* locus (Steinmann-Zwicky, 1988). At the molecular level, the sex-specific splicing of *Sxl* pre-mRNAs has been analyzed in 2X:2A germ cells apparently transformed to spermatocytes because of inappropriate somatic signal or because they were homozygous for the snf^{1621}, otu^{1} or fu^{1} mutations (B. Oliver, Y.-J. Kim and B. S. Baker, unpublished data). Using reverse transcription followed by amplification by polymerase chain reaction (RT-PCR) as well as in situ hybridization with the male-specific exon, they showed that the *Sxl* transcripts are spliced in the male-specific mode in transformed germ cells. Here we have used RT-PCR to extend these observations to additional *otu* alleles.

Fig. 3A shows the simplified structure of *Sxl* pre-mRNAs and the male and female mode of splicing. The male mode of processing maintains a small exon containing a stop codon which interrupts the open reading and leads to the production of short non-functional peptides. A primer within the male exon and a non-sex-specific upstream primer were used specifically to detect transcripts spliced along the male mode. Fig. 3B shows a RT-PCR experiment with mRNAs extracted from ovaries or carcasses (gonadectomized flies) of females homozygous for otu^{1}, otu^{7} or otu^{8}. For all three mutations (as well as otu^{10}, data not shown), *Sxl* male exon sequences were amplified from mRNAs extracted from the gonads, but not from somatic cell mRNAs or from mRNAs extracted from wild-type ovaries. This observation suggests that alteration of *Sxl* pre-mRNAs splicing is a general effect of *otu* mutations though it does not prove that the *otu* gene directly regulates the processing of *Sxl* transcripts.

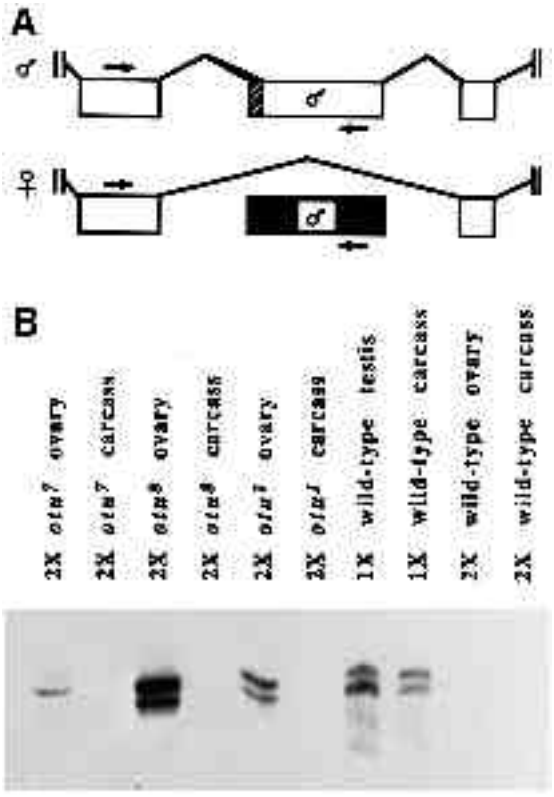

Fig. 3. Alteration of splicing of *Sxl* pre-mRNAs by *otu* mutations. (A) Male and female splicing patterns around the male-specific exon of *Sxl* pre-mRNAs. The two primers used for PCR amplification of the male-specific exon are indicated by arrows. (B) Relevant portion of an autoradiogram of a 5% polyacrylamide sequencing gel used to separate the two male-specific fragments. 1X and 2X refer to the number of X chromosomes. 1X flies were somatically male and 2X were somatically female. RNAs were extracted from the gonads or the carcasses (gonadectomized flies) of females homozygous for the otu^{7}, otu^{8} or otu^{1} mutations, as well as those of wild-type males and females.

Partial suppression of the sexual transformation by constitutive expression of the *Sex-lethal* gene

One prediction that ensues from the results described above is that the expression of the feminizing activity of *Sxl* should lead to suppression of the transformed sexual identity of 2X:2A ; *otu*⁻ germ cells. Previously, the constitutive mutation Sxl^{M1} has been used to show that *Sxl* acts downstream of the snf^{1621} mutation in germ cells (Steinmann-Zwicky, 1988). In an attempt to determine the position of the *otu* locus relative to *Sxl*, we tested the ability of Sxl^{M1} to affect the phenotype of some *otu* alleles. We describe as rescue (suppression) the disappearance of male-like germ cells, and as partial rescue a strong reduction of the abundance of male-like germ cells.

Different results were obtained depending on the combination of *otu* alleles used. They are summarized in Table 1. For instance, combinations of otu^{1} with otu^{1}, otu^{3}, otu^{11} or otu^{13} were rescued or partially rescued by Sxl^{M1}. The disappearance of transformed germ cells was accompanied by increased numbers of egg chambers characteristic of weak *otu* alleles, e.g. small oocytes whose chorion is opened at the anterior (Fig. 4B) or abnormal number of nurse cells.

Fig. 2. Expression of male-specific reporter genes in transformed 2X:2A germ cells. Gonads were processed as described in Materials and methods. (A) Nomarski view of the tip of a testis showing β-galactosidase expression in stem cells and in the mitotic region. Note the absence of expression in fully grown spermatocytes. Genotype: otu^{1} / Y ; P[w^{+} lacZ]606 / P[w^{+} lacZ]606. (B) Bright-field view of normal egg chambers from germarium to stage 8 or 9. Genotype: otu^{1} / *FM7a*, otu^{+} ; P[w^{+} lacZ]606 / P[w^{+} lacZ]606. (C) Bright-field view of otu^{11} / otu^{11} ; P[w^{+} lacZ]590 / P[w^{+} lacZ]590 ovaries. The egg chambers are full of male-like germ cells that express β-galactosidase. (D) Nomarski view of an otu^{1} / otu^{1} ; P[w^{+} lacZ]606 / P[w^{+} lacZ]606 egg chamber. Note the absence of staining of follicle cells (somatic origin) and of the few nurse cells (female differentiation) at the tip of the chamber. The arrowhead points to a nurse cell nucleus. Bar, 25 μm (A, D); 50 μm (B), or 100 μm (C).

Table 1. Effect of constitutive expression of feminizing functions of *Sxl* on the sexual identity of *otu*⁻ germ cells

	otu^{1}	*otu*^{2}	*otu*^{3}	*otu*^{6}	*otu*^{7}	*otu*^{9}	*otu*^{10}	*otu*^{11}	*otu*^{13}	*otu*^{17}
Sxl^{M1} *otu*^{1} (1)	+	−	+	−	NA	ND	−	++*	++*	−
Sxl^{M1} *otu*^{1} (28)	++	−	++	−	NA	+	−	++*	++*	−
Sxl^{M1} *otu*^{9}	−/+	−	++	−	ND	+	+	−	+	−
Sxl^{M1} *otu*^{10}	−/+	++	−	−	+	−/+	−	++*	−	−
Sxl^{M1} *otu*^{11}	−/+	ND	+	−	−	−/+	−	−/+	−	−/+

Females of genotype *Sxl*^{M1} *otu*^{x} / *Sxl*$^{+}$ *otu*^{y} were aged for 7 days before dissection. The abundance of male-like germ cells was compared to that observed in absence of constitutive expression of *Sxl* (females of genotype *Sxl*$^{+}$ *otu*^{x} / *Sxl*$^{+}$ *otu*^{y}).

−, no suppression of the sexual transformation of *otu*⁻ germ cells by constitutive activity of *Sxl*.

−/+, no suppression or partial suppression in some ovaries.

+, partial suppression (strong reduction in the frequency of male-like germ cells.

++, complete suppression (absence of the expected male-like germ cells).

NA, not applicable (the control ovaries contain no or very few male-like cells. ND, not done.

(1) and (28) are two independent recombinant chromosomes bearing the *Sxl*^{M1} and *otu*^{1} mutations. Note that the effect of *Sxl*^{M1} seems to be sensitive to genetic background since the two recombinants gave slightly different suppression at least when combined with specific *otu* alleles.

*Fertility was improved (see text).

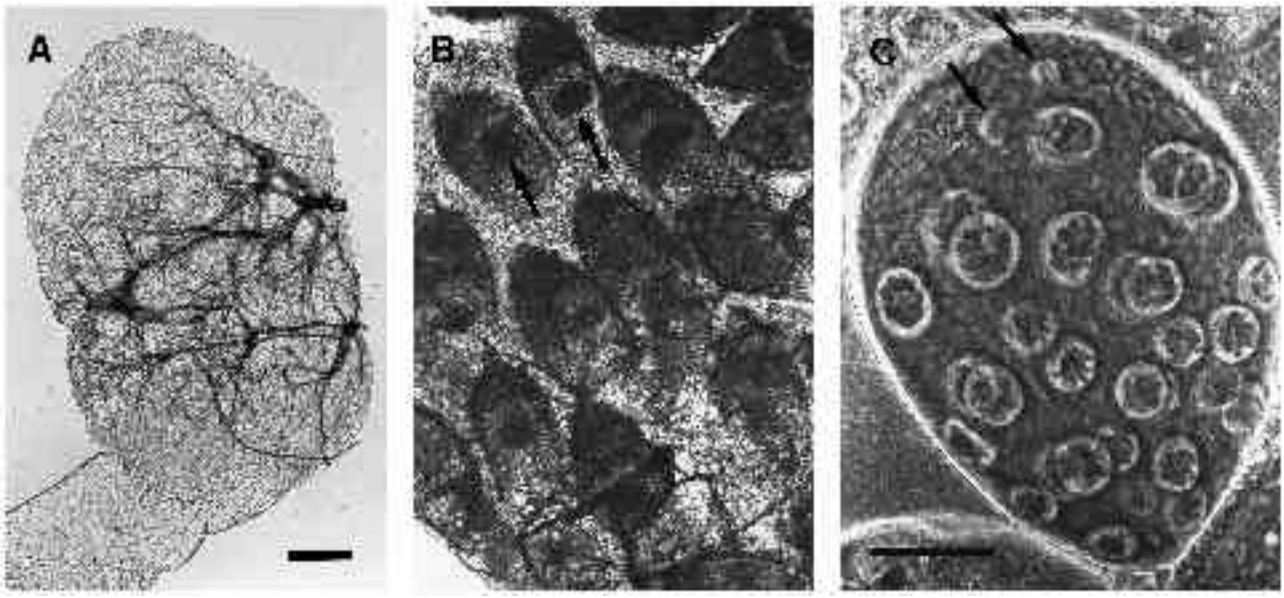

Fig. 4. Suppression of the sexual transformation of 2X:2A ; *otu*⁻ germ cells by constitutive expression of feminizing activity of *Sex-lethal*. (A) Low magnification of an *otu*^{1} / *otu*^{1} ovary containing mostly transformed germ cells such as those shown in Fig. 1. Bar, 100 μm. (B) A complete suppression of the transformed phenotype due to the presence of the *Sxl*^{M1} allele. Genotype: *Sxl*^{M1} *otu*^{1} (28) / *Sxl*$^{+}$ *otu*^{1}. The oocytes are small, the anterior end of their chorion is opened and the dorsal appendages are reduced to a fused spot in an abnormal posterior position (arrows). Same magnification as (A). (C) An example of egg chamber with an excessive number of germ cells that differentiate along the female pathway. Genotype as in (B). Note the presence of two oocyte nuclei (arrows) and 30 nurse cells. The accumulation of some yolk near the oocyte nuclei indicates that this egg chamber proceeded up to stage 8 of oogenesis. Bar, 50 μm.

One striking example of the latter are egg chambers containing 2 oocytes and 30 nurse cells (Fig. 4C), that is twice the normal numbers. The abundance of egg chambers with excess number of nurse cells strongly indicates that in addition to its essential role in the determination of the sexual identity of germ cells, *otu* plays a critical role in the regulation of cystocyte divisions, a function that has been proposed previously (King et al., 1986).

In some instances, fertility was either partially restored (*Sxl*^{M1} *otu*^{1} / *Sxl*$^{+}$ *otu*^{13}: 17 fertile females out of 17 tested, producing more than 10 progeny per female in 10 days; control: 1 fertile out of 20, with less than 10 progeny) or improved (*Sxl*^{M1} *otu*^{1} / *Sxl*$^{+}$ *otu*^{11}: 18 fertile out of 20, with more than 10-20 progeny per fertile female in 10 days; control: 40 fertile out of 52, but with fewer than 10 progeny per fertile female).

Sxl^{M1} had no effect on the phenotype of combinations of *otu*^{1} with strong *otu* alleles such as *otu*^{2}, *otu*^{6}, *otu*^{8}, *otu*^{10} or *otu*^{17}. For other recombinants that were analyzed (*Sxl*^{M1} *otu*^{9}, *Sxl*^{M1} *otu*^{10} and *Sxl*^{M1} *otu*^{11}), the constitutive expression of *Sxl* had usually no or weak effects. The absence of rescue for combinations of strong mutations is not unexpected since constitutive activity of *Sxl* should have no effect if the defect (germ cell death or quiescence of the stem cells) takes place before the time when *Sxl* is required. Since *Sxl*^{M1} is not a completely constitutive mutation

Table 2. Enhancement of the *ovo^D* ovarian phenotype by *otu* mutations

Genotype of females		*ovo^{D2}*				*ovo^{D3}*			
		Oocytes/ovary		Statistics		Oocytes/ovary		Statistics	
Parent	Progeny	Mean	No.	T1	*P*	Mean	No.	T1	*P*
ct otu^1 v /	*ovo^D/otu^1*	0.000	100	0.300	<0.01	0.015	100	0.857	<0.01
FM7a (onc)	*ovo^D/FM7a*	0.639	90			5.472	90		
y cv otu^2 v f	*ovo^D/otu^2*	0.075	80	0.950	<0.01	0.180	75	1.000	<0.01
/ *FM3* (qui)	*ovo^D/FM3*	9.164	61			9.421	63		
otu^3 v / *FM3*	*ovo^D/otu^3*	0.025	60	0.236	NS	0.804	102	0.450	<0.01
(onc)	*ovo^D/FM3*	0.402	87			2.130	81		
otu^6 / *FM7c*	*ovo^D/otu^6*	0.000	60	0.400	<0.01	0.212	78	0.796	<0.01
(qui)	*ovo^D/FM7c*	0.900	50			5.246	63		
otu^7 / *FM7*	*ovo^D/otu^7*	0.173	52	0.492	<0.01	0.477	66	0.758	<0.01
(dif)	*ovo^D/FM7*	3.214	28			6.129	62		
otu^8 / *FM3*	*ovo^D/otu^8*	0.083	54	0.917	<0.01	0.357	70	0.971	<0.01
(qui)	*ovo^D/FM3*	9.240	48			12.179	70		
y cv otu^9 v f	*ovo^D/otu^9*	0.031	48	0.717	<0.01	0.344	77	1.000	<0.01
/ *FM0* (dif)	*ovo^D/FM0*	3.631	42			14.099	81		
y cv otu^{10} v f	*ovo^D/otu^{10}*	0.000	124	0.880	<0.01	0.068	109	0.976	<0.01
/ *FM0* (qui)	*ovo^D/FM0*	3.533	75			11.187	123		
y cv otu^{11} v f	*ovo^D/otu^{11}*	0.052	86	0.912	<0.01	8.046	54	0.600	<0.01
/ *FM3* (onc)	*ovo^D/FM3*	5.825	57			15.317	41		
y cv otu^{13} v f	*ovo^D/otu^{13}*	0.000	40	1.000	<0.01	0.118	38	1.000	<0.01
/ *FM0* (onc)	*ovo^D/FM0*	6.463	43			9.625	44		
otu^{14} / *FM7*	*ovo^D/otu^{14}*	0.080	56	0.066	NS	2.311	74	0.177	NS
(dif)	*ovo^D/FM7*	0.220	50			3.188	64		
y w otu^{17} /	*ovo^D/otu^{17}*	0.000	106	0.480	<0.01	0.000	110	0.897	<0.01
FM7 (qui)	*ovo^D/FM7*	1.500	50			5.581	68		
otu^{Pd4} / *FM7*	*ovo^D/otu^{Pd4}*	0.048	124	0.680	<0.01	0.204	108	0.943	<0.01
(qui)	*ovo^D/FM7*	2.983	118			9.819	80		

Female parents of the indicated genotype were crossed to *ovo^{D2} v^{24}* / Y or *ovo^{D3} v^{24}* / Y males. Progeny genotypes were assigned on the basis of the *Bar* eye phenotype associated with the balancer chromosomes.

Mean, the mean number of egg chambers at stage 10 or more advanced per ovary.

No., the number of ovaries scored. A non-parametric Smirnov test (Connover, 1980) was used. T1 is the maximal distance between cumulative distributions of eggs/ovary for *otu* females compared to their balancer siblings. T1=1.000 means that all the experimental ovaries have less eggs than any control ovaries. T1=0.000 means that there is no difference between control and experimental ovaries.

P , level of significance; NS, not significant.

The strength of the *otu* alleles indicated in parenthesis after the parent genotype is according to the classification of King et al. (1986): qui, quiescent; onc, oncogenic; and dif, defective in late oogenic development.

(Steinmann-Zwicky 1988), we have also tested the effect of the stronger constitutive allele, *Sxl^{M4}*, but no major difference was found (data not shown).

Our results indicate that the *otu* gene does not act in a single step during oogenesis with regards to the *Sxl* gene. However, the suppression (partial suppression in some allelic combinations) of the ovarian tumor phenotype, as well as the alteration of *Sxl* pre-mRNA splicing described above, clearly show that *otu* acts upstream of *Sxl* in the process of determination of the sexual identity of the germ line.

Genetic interactions suggest that *otu* and *ovo* act in the same pathway

Three dominant antimorphic alleles of *ovo*, *ovo^{D1}*, *ovo^{D2}* and *ovo^{D3}*, have been isolated (Busson et al., 1983). In heterozygous flies, the strongest allele, *ovo^{D1}*, reduces the viability of female germ cells and arrests oogenesis around stage 4 (Perrimon, 1984), although more advanced previtellogenic stages can occasionally be found (data not shown). In *ovo^{D2}/ovo$^+$* females, oogenesis usually stops around stage 6 with only a few defective vitellogenic oocytes. The number of vitellogenic *ovo^{D2}/ovo$^+$* oocytes depends on the genetic background (Oliver et al., 1990). Although totally sterile, *ovo^{D3}/ovo$^+$* ovaries are close to wild-type in size and contain many eggs, which appear almost normal except for the permeability of their vitelline membrane and the occasional fusion of their dorsal appendages. Phenotypes of *ovo^D/ovo$^+$* ovaries are modified in flies that are also heterozygous for some alleles of *Sxl* or for *snf^{1621}* (Oliver et al., 1990).

Thirteen *otu* alleles, ranging from very strong (quiescent), intermediate (oncogenic, tumorous ovaries), to weak (differentiation-defective; King et al., 1986; Storto and King, 1987; 1988), were tested for dominant interaction with *ovo^D* mutations. Table 2 shows that most alleles strongly interact with either *ovo^{D2}* or *ovo^{D3}*, resulting in a more extreme mutant phenotype. Interaction was quantified by counting the number of late vitellogenic oocytes of stages 10-14 in flies double heterozygous for *ovo^D* and *otu^x* (*ovo^D otu$^+$/ ovo$^+$ otu^x*) compared to their sibling (*ovo^D otu$^+$/ ovo$^+$ otu$^+$*). No or very few oocytes (1-2 per 10 ovaries with *ovo^{D2}* and 1-8 per 10 ovaries with *ovo^{D3}*) were found in double heterozygotes compared to several oocytes in control flies (4-92 per 10 ovaries with *ovo^{D2}* and 55-141 per 10 ovaries with *ovo^{D3}*). We could not find any obvious correlation between the strength of an *otu* allele and its interaction with *ovo^{D2}* or *ovo^{D3}*. This is not entirely surprising since these two *ovo^D* mutations are very sensitive to changes of the genetic background (Oliver et al., 1990; D.P., unpublished data): even a

Table 3. Dependence of *ovo^D* ovarian phenotype on the number of *otu$^+$* genes

(A)

Genotype of females		*ovo^{D2}*				*ovo^{D3}*			
		Oocytes/ovary		Statistics		Oocytes/ovary		Statistics	
Parent	Progeny	Mean	No.	T1	*P*	Mean	No.	T1	*P*
Df(1)RA2 /	*ovo^D/Df*	0.000	112	0.393	<0.01	0.044	68	0.826	<0.01
FM7c	*ovo^D/FM7c*	0.949	107			4.051	69		
Df(1)KA14 /	*ovo^D/Df*	0.034	89	0.945	<0.01	0.500	54	0.940	<0.01
FM7c	*ovo^D/FM7c*	9.890	91			12.403	67		
Df(1)KA14 /	*ovo^D/Df*	0.000	60	0.900	<0.01	ND			
FM6	*ovo^D/FM6*	7.710	50			ND			

(B)

Genotype of females		Number of *otu$^+$* copies	*ovo^{D2}* Oocytes/ovary		*ovo^{D3}* Oocytes/ovary	
Parent	Progeny		Mean	No.	Mean	No.
Df(1)RA2/FM7a;	*ovo^D/Df;CyO/+*	1	0.000	74	0.194	85
Dp(1;2)FN107/CyO	*ovo^D/Df;Dp/+*	2	1.610	73	8.635	78
	ovo^D/FM7a;CyO/+	2	0.700	80	6.455	77
	ovo^D/FM7a;Dp/+	3	7.481	79	12.966	74

See Table 2 for details. ND, not done. The *CyO* balancer chromosome was recognized by the dominant wing phenotype *Cy*. The differences between one, two or three *otu$^+$* genes are all highly significant.

slight reduction of *otu$^+$* activity might be sufficient to give a strong interaction. The only exception was *otu^{14}*, which showed no significant interaction with either *ovo^{D2}* or *ovo^{D3}*. This allele is the weakest *otu* mutation so far isolated (King et al., 1986). The *otu^{14}* allele produces a polypeptide truncated for the last 156 amino acids (Steinhauer et al. 1989; Steinhauer and Kalfayan 1992), which clearly retains much activity and is not defective in the early function of sex determination.

The *otu* locus is uncovered by two overlapping deletions, *Df(1)RA2* (7D10;8A4-5) and *Df(1)KA14* (7F1-2;8C6). They were used to determine whether the interaction observed between *ovo^{D2}* or *ovo^{D3}* and the *otu* alleles can be obtained simply by changing the dose of *otu$^+$* activity. Table 3A summarizes the interaction observed between *ovo^{D2}* or *ovo^{D3}* and the two deletions uncovering *otu*. With *Df(1)RA2* and *ovo^{D2}* no vitellogenic oocyte was observed whereas a single egg was found in 89 ovaries of genotype *ovo$^+$ Df(1)KA14* / *ovo^{D2} Df$^+$*). These numbers are significantly different from those observed in control siblings. A few vitellogenic stages were observed when *ovo^{D3}* was used. These results are in full agreement with those obtained with most *otu* alleles and are probably due entirely to the dosage of *otu$^+$* activity since none of the mutations that we tested in nine lethal complementation units uncovered by the deletions showed any interaction with *ovo^{D2}* and *ovo^{D3}* (not shown). Moreover, these results indicate that the dominant interaction is dose dependent and does not require the presence of mutant otu proteins.

Dose dependency was further tested by using a parental stock that bears *Df(1)RA2* and a duplication of the region (*Dp(1;2)FN107*, 7A8 to 8A5 inserted on the 2nd chromosome). Four different genotypes were recovered in the female progeny. They differ in the number of copies of region 7E-8A (Table 3B). The two genotypes with two copies of the region produced similar numbers of vitellogenic eggs per ovary. These numbers are within the range observed in various wild-type backgrounds (Oliver et al., 1990; D.P. unpublished data). As described above, a single copy of the region strongly reduced the number of vitellogenic oocytes per ovary (0.00 in *trans* with *ovo^{D2}* and 0.194 with *ovo^{D3}*). In contrast, three copies of the region improved the *ovo^{D2}* and *ovo^{D3}* phenotypes. Many mature oocytes were produced and they were less defective (less flaccid and lower frequency of fusion of the dorsal appendages) than oocytes produced by females bearing two copies of the region. Furthermore, absence of any maternal effect is suggested by the fact that no difference was observed whether the mother had 1 or 2 *otu$^+$* genes (compare *ovo^D Df$^+$/ovo$^+$ Df(1)RA2* in Table 3A with *ovo^D Df$^+$/ovo$^+$ Df(1)RA2;CyO/+* in Table 3B). In summary, one dose of *otu$^+$* enhances the *ovo^{D2}* and *ovo^{D3}* phenotypes whereas three doses acts as suppressor.

The two deletions and the *otu* alleles were also tested in *trans* with *ovo^{D1}*. At first glance, hemizygosity for region 7F-8A or heterozygosity at the *otu* locus produced little modification of the *ovo^{D1}* ovarian phenotype: comparable numbers of pre-stage 4 egg chambers and no advanced stages were observed in the presence of either one or two *otu$^+$* copies. However, in ovaries with only one wild-type *otu* gene, exceptional egg chambers were found at a frequency of about one per ovary. These egg chambers contained a few germ cells whose morphology was similar to that of spermatocytes (Fig. 5). This sexual transformation is similar to that observed by interaction between *ovo^{D1}* and *snf^{1621}*, except that the latter occurred at a higher frequency and the transformed egg chambers contained more germ cells (Oliver et al., 1990).

In summary, dominant interactions were found between the three *ovo^D* alleles and most *otu* alleles. First, the ovarian

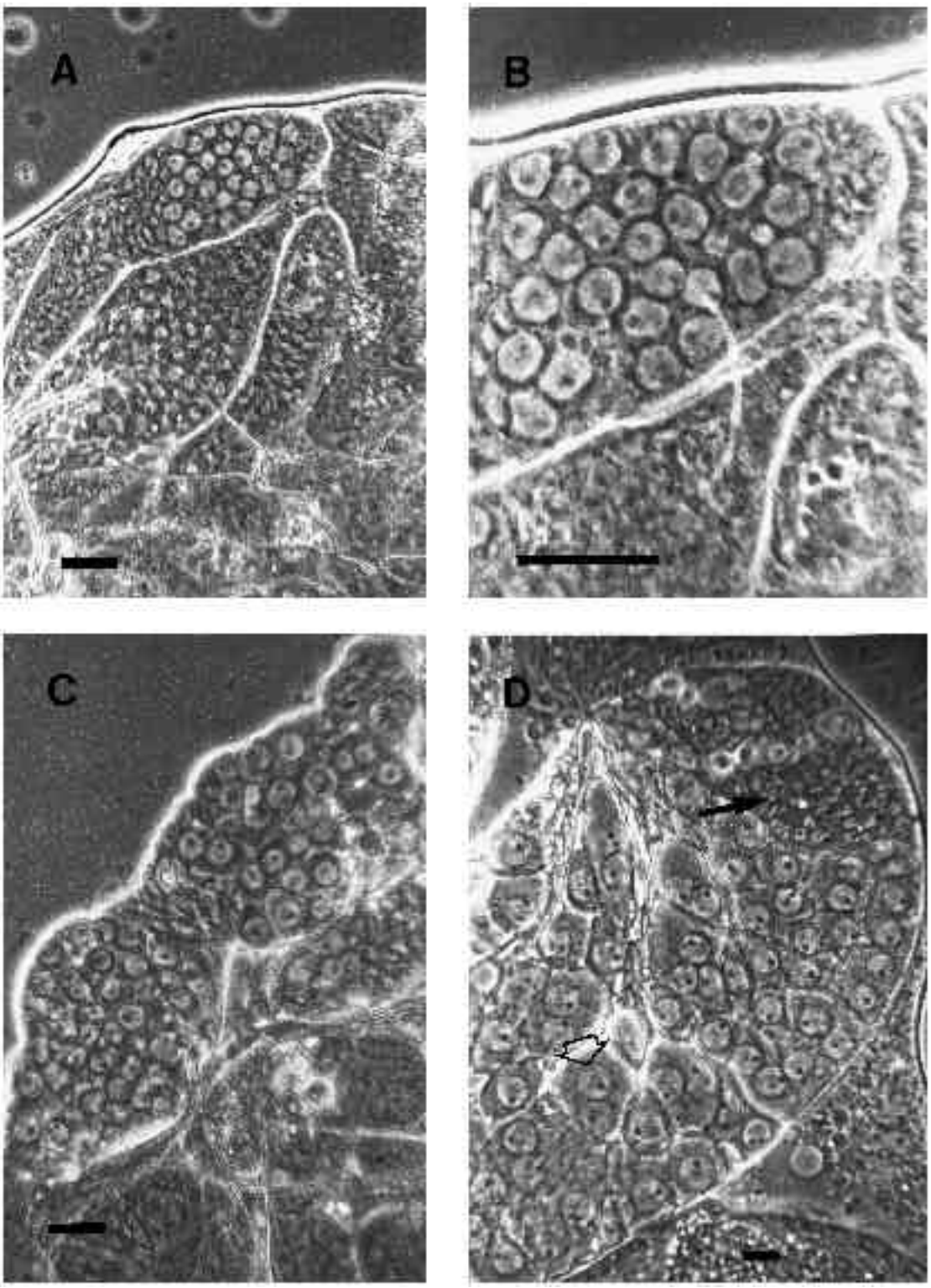

Fig. 5. Synergistic interaction between ovo^{D1} and *otu* alleles. (A) Ovary with an egg chamber containing germ cells that resemble spermatocytes. Genotype: ovo^{D1} otu^{+} / ovo^{+} otu^{13}. Note that the transformed germ cells are smaller than mature spermatocytes. (B) Higher magnification of (A). (C) Another example of transformed egg chambers. Genotype: ovo^{D1} otu^{+} / ovo^{+} otu^{11}. Magnification as in (A). (D) Tip of a testis showing the stages of mitotic proliferation (arrow) and mature spermatocytes (open arrow). Genotype: *FM7a* / Y. Bars, 20 μm.

phenotypes of ovo^{D2}/+ and ovo^{D3}/+ flies are dependent on the dose of the otu^{+} gene. Second, synergistic interaction was found between *otu* alleles and ovo^{D1}, leading to the sexual transformation of some 2X:2A germ cells towards male differentiation. These interactions are similar to those observed between ovo^{D} and *snf*, another germ line sex determination locus.

DISCUSSION

Mutations in genes required in germ cells for proper determination of their sexual identity have been shown to produce two phenotypes: (1) sex-specific death or quiescence of the stem cells and (2) differentiation of germ cells of one chromosomal constitution along the developmental pathway of the other sex. In this paper, we present several lines of evidence that the *otu* locus, whose mutations can show either phenotype, is involved in germ line sex determination.

Strong loss-of-function alleles of *otu*, like the absence of *ovo* activity, cause the absence of stem cells or their complete quiescence in the female gonad (Storto and King, 1988). In partial loss-of-function mutations, females germ cells differentiate but their morphology resembles young spermatocytes. Since the morphology of small germ cells is a phenotype difficult to analyze without some ambiguity, we have used molecular markers to assess the sex of otu^{-} germ cells. We have shown that two *lacZ* reporter genes normally active in the male but not in the female germ line are expressed in 2X:2A ; otu^{-} germ cells. This observation unambiguously demonstrates that these germ cells have adopted a male sexual identity. The same result has been obtained with *Sxl* and *snf* mutations (D. P., unpublished data). Expression in ovarian tumors of other male-specific genes, for instance *Stellate*, further supports the view that chromosomally female germ cells differentiate along the male pathway when mutant for the *Sxl* or *snf* genes (G. Wei, B. Oliver and A. P. Mahowald, unpublished data). Although they might be controlled by the same enhancer (P. Gönczy, S. DiNardo and D. Pauli, unpublished data) the two reporter genes described in this paper should be very useful for determining whether other mutations (*bgcn*, *fes*, *fu*, see Lindsley and Zimm, 1992 for detailed description of these three loci; *bam*, McKearin and Spradling, 1990; *fl(2)d*, Granadino et al., 1992), the phenotype of which has been described as tumorous ovary, actually change the sexual identity of 2X:2A germ cells.

Except during early embryogenesis (Salz et al., 1989; Keyes et al., 1992), the sex-specific differential expression of *Sxl* is regulated at the level of pre-mRNA splicing. This is true for somatic and germinal cells (Bell et al., 1988; Bopp et al., 1991; Samuels et al., 1991; B. Oliver, Y.-J. Kim and B. B. Baker, unpublished data). In males, an additional exon, containing in frame stop codons, is incorporated into mature mRNAs, leading to the absence of functional Sxl polypeptides. The processing of *Sxl* transcripts in the male mode described in this paper for several *otu* mutants is similar to the alteration of *Sxl* pre-mRNA splicing observed in snf^{1621}, fu^{1} or otu^{1} transformed germ cells (B. Oliver, Y.-J. Kim and B. S. Baker, unpublished data). Similar processing has also been observed when an inappropriate somatic signal was provided, because of mutations in the *tra*, *tra-2* and *dsx* genes, which transform chromosomal (2X:2A) females into phenotypic males (B. Oliver, Y.-J. Kim and B. S. Baker, unpublished data). Mutations of the *fl(2)d* locus have also been shown to affect the splicing of *Sxl* transcripts (Granadino et al., 1990). Obviously these results do not prove a direct role of any of these genes in the regulation of *Sxl* pre-mRNAs splicing, but they indicate that these genes either participate in a single regulatory pathway upstream of *Sxl* or are parts of parallel pathways which come together to control the expression of *Sxl*.

Mutations of *Sxl* that constitutively provide the feminizing activity of the gene have been used to demonstrate that *snf* (Steinmann-Zwicky, 1988; Salz, 1992), *fl(2)d* (Granadino et al., 1992) and *ovo* (B. Oliver, Y.-J. Kim and B. S. Baker, unpublished data) act upstream of *Sxl*, in agreement with the molecular data summarized above. The effect of two constitutive alleles of *Sxl* was studied in females bearing various heteroallelic combinations of *otu* mutations. We found that constitutive *Sxl* activity was efficient at improving one of the *otu* phenotypes, that is the ovarian tumor phenotype. In several cases, the frequency of sexual transformation of 2X:2A germ cells towards spermatocytes was strongly reduced. This was especially true with alleles such as otu^{1}, otu^{11} or otu^{13} whose primary phenotype is tumorous ovaries. The fertility of some heteroallelic combinations (otu^{1}/otu^{11} or otu^{1}/otu^{13}) was also improved, but this is probably a consequence of the increased number of germ cells that differentiate along the female pathway.

Constitutive *Sxl* activity does not seem to rescue the germ cell death or early arrest of strong *otu* alleles. Similarly, Sxl^{M1} has no effect on strong ovo^{-} mutations that kill 2X:2A stem cells (Steinmann-Zwicky et al., 1989; Oliver et al., 1990). This suggests that *Sxl* is probably not involved in the establishment of the early dimorphism that has been observed between male and female germ cells (Oliver et al., 1987; Wei et al., 1991). Absence of an early function of *Sxl* in the germ line is supported by molecular experiments that failed to detect expression of Sxl proteins in embryonic germ cells (Bopp et al., 1991). It is not known why 2X:2A germ cells die when they do not have any *ovo* or *otu* genes activity. Based on what is known for somatic cells, our best hypothesis is that female germ cell death is due to failure of dosage compensation, a process necessary to equalize the activity of X-linked genes in male (1X) and female (2X) (Lucchesi and Manning, 1987). No direct evidence for dosage compensation has been found so far in the germ line (see Schüpbach, 1985; Bachiller and Sanchez, 1986), but it would be surprising if germ cells could survive in the absence of this important process.

The dominant female sterile alleles of the *ovo* locus have been shown to interact with mutations of the *Sxl* and *snf* genes (Oliver et al., 1990). In this paper, we have shown that *otu* mutations behaved like the snf^{1621} allele. In flies doubly heterozygous for ovo^{D1} and either *snf* or *otu*, a synergistic interaction led to the differentiation of a few 2X:2A germ cells along the male pathway. This is strong evidence for the involvement of the three genes in germ line sex determination. With ovo^{D2} and ovo^{D3}, a clear dosage effect was

observed: decreasing the number of wild-type *otu* genes enhances the ovo^D ovarian phenotype, while increasing the dose of otu^+ had a suppressor effect (though fertility was not restored). In other words, *otu* gene products appear to be rate limiting factors in ovo^{D2} and ovo^{D3} ovaries. We would like to stress that the interaction with ovo^{D2} and ovo^{D3} probably takes place at oogenic steps later than sex determination because all the germ cells are female in those ovaries. This is consistent with the existence of multiple functions for both *ovo* and *otu*, as deduced from the phenotypes of hypomorphic alleles.

Our study clearly shows that one of the roles of *otu* is in germ line sex determination. How this function relates to previously suggested roles is not clear. Other roles range from the control of cystocyte divisions (initiation and control of the number of mitoses), to the choice between differentiation as oocyte or as nurse cell, to the dumping of nurse cells cytoplasm in the maturing oocytes. Additional functions in the control of nurse cell nuclei polyploidization and the synthesis of beta yolk spheres have been suggested (King et al., 1986; Storto and King, 1987; 1988; Tirronen et al., 1992). We found many egg chambers containing supernumerary nurse cells when the sexual identity of otu^- germ cells was channeled along the female pathway by constitutive expression of *Sxl*. This observation is consistent with the idea that after its role in germ line sex determination, *otu* has a function in the control of mitosis of female germ cells. Alternatively, abnormal numbers of germ cells per egg chamber could be due to failure of the follicle cells to enclose the 16 cystocytes as found in *brn* mutations (Goode et al. 1992).

Molecular studies have shown that the *otu* gene encodes two proteins. The larger polypeptide contains an extra 42 amino acids in its middle and appears to be expressed before cystocyte differentiation (Steinhauer and Kalfayan, 1992). The smaller polypeptide seems to be expressed later during oogenesis. Two mutations producing mainly ovarian tumors and which are partially suppressed by constitutive expression of *Sxl* have been single base pair substitutions affecting the additional exon. In otu^{11}, it leads to an amino acid substitution while in otu^{13} no large polypeptide is produced due to a splice site mutation (Steinhauer and Kalfayan, 1992). Assuming that the two otu proteins have different non-redundant functions, only the large polypeptide is expressed early enough to be involved in germ line sex determination. We would expect that expression of the large protein should be sufficient to rescue the ovarian tumor phenotype of *otu* mutations. If this is the case, constitutive expression of the large polypeptide should help to determine the position of *otu* compared to *ovo*, *snf*, *fu* and *fl(2)d*. It would also be interesting to know whether the large otu protein is capable, like constitutive expression of *Sxl* (Steinmann-Zwicky et al., 1989), of overriding the sexual transformation observed when 2X:2A pole cells are transplanted into a male soma.

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