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PAULI, Daniel, OLIVER, Brian, MAHOWALD, Anthony P.

Abstract

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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 spermatoocytes 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 ovoD ovarian phenotypes, while three doses partially suppress these phenotypes. Synergistic interaction between ovoD and otu alleles leads to the occasional transformation of chromosomally female germ cells into early spermatocytes. These interactions are similar to those observed between ovoD 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 otu10 have no, or few, female germ cells, which fail to differentiate. Several milder alleles like otu1 or otu13 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 snf1621 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 (ovoD) were analyzed as described below. Briefly, virgin females were crossed to ovoD 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 ovoD8 and ovoD3 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 ovoD and the mutation to be tested were compared to their siblings heterozygous for ovoD. A non-parametric Smirnov statistical test (Conover, 1980) was used to analyze the data.

To test the effect of constitutive expression of Sex-lethal, recombinant chromosomes were generated between SxlM1 or SxlM4 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/d+ females (Cline, 1978). The rescuing effect of constitutive expression of Sxl was analyzed in females of genotypes SxlM1 otu+ / Sxl+ otu− Control flies (Sxl+ otu+ / Sxl+ otu+) 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 NaH2PO4/Na2HPO4 pH 7.2, 1% MgCl2, 150 mM NaCl, 5 mM K4[Fe(CN)6] and 5 mM K3[Fe(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.,
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 $^{32}$PdATP (Saiki et al., 1988). To detect only the male-specific mode of splicing, the following primers were used: non-sex-specific 5′ ACAACGACAGCAGGCCA (position 286-305, Bell et al., 1988) and specific for the male exon 5′ GGGCTTTGGAGGTGTCCTCG (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 spermatogene-

sis 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 phe-notypically normal germ line of heterozygous females or in somatic follicle cells (Fig. 2B). In contrast, strong staining was observed in ovaries homozygous for otu1, otu3, otu11 or otu13, 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: otu1 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. 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: otu1 / otu1. (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.
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.

Altering the splicing of Sex-lethal transcripts by otu mutations

Genetic evidence indicates that in germ cells the Sex-lethal 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 Sex-lethal 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 snf1621, otu1 or fu1 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 Sex-lethal 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 Sex-lethal 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 otu1, otu3 or otu8. For all three mutations (as well as otu10, data not shown), Sex-lethal 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 Sex-lethal pre-mRNAs splicing is a general effect of otu mutations though it does not prove that the otu gene directly regulates the processing of Sex-lethal transcripts.

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 Sex-lethal should lead to suppression of the transformed sexual identity of 2X:2A; otu− germ cells. Previously, the constitutive mutation Sex-lethalM1 has been used to show that Sex-lethal acts downstream of the snf1621 mutation in germ cells (Steinmann-Zwicky, 1988). In an attempt to determine the position of the otu locus relative to Sex-lethal, we tested the ability of Sex-lethalM1 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 otu1 with otu1, otu3 or otu11 were rescued or partially rescued by Sex-lethalM1. 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.
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 male-like germ cells was compared to that observed in absence of constitutive expression of Sxl (females of genotype Sxl<sup>M1</sup> o<sup>tu</sup> / Sxl<sup>M1</sup> o<sup>tu</sup>). –, no suppression of the sexual transformation of o<sup>tu</sup> germ cells by constitutive activity of Sxl.

In some instances, fertility was either partially restored (Sxl<sup>M1</sup> o<sup>tu</sup> / Sxl<sup>M1</sup> o<sup>tu</sup>; 17 fertile females out of 17 tested, producing more than 10-20 progeny per fertile female in 10 days; control: 1 fertile out of 20, with less than 10 progeny) or improved (Sxl<sup>M1</sup> o<sup>tu</sup> / Sxl<sup>M1</sup> o<sup>tu</sup>; 18 fertile out of 20, with more than 10-20 progeny per fertile female in 10 days).

Sxl<sup>M1</sup> had no effect on the phenotype of combinations of o<sup>tu</sup> with strong o<sup>tu</sup> alleles such as o<sup>tu</sup>, o<sup>tu</sup>, o<sup>tu</sup>, o<sup>tu</sup> or o<sup>tu</sup>“. For other recombinants that were analyzed (Sxl<sup>M1</sup> o<sup>tu</sup>, Sxl<sup>M1</sup> o<sup>tu</sup> and Sxl<sup>M1</sup> o<sup>tu</sup>), 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<sup>M1</sup> is not a completely constitutive mutation

Females of genotype Sxl<sup>M1</sup> o<sup>tu</sup> / Sxl<sup>M1</sup> o<sup>tu</sup> 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<sup>M1</sup> o<sup>tu</sup> / Sxl<sup>M1</sup> o<sup>tu</sup>.

<table>
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<th>o&lt;sup&gt;tu&lt;/sup&gt;</th>
<th>o&lt;sup&gt;tu&lt;/sup&gt;</th>
<th>o&lt;sup&gt;tu&lt;/sup&gt;</th>
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<td>++*</td>
</tr>
<tr>
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<td>–</td>
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<td>–</td>
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<td>–</td>
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<td>++</td>
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<tr>
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<td>–/+</td>
<td>ND</td>
<td>+</td>
<td>–</td>
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</table>

*Fertility was improved (see text).

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Table 1. Effect of constitutive expression of feminizing functions of Sxl on the sexual identity of o<sup>tu</sup>– germ cells

<table>
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<tr>
<th>Allele</th>
<th>o&lt;sup&gt;tu&lt;/sup&gt;</th>
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</tbody>
</table>

++/+, 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<sup>M1</sup> and o<sup>tu</sup> mutations. Note that the effect of Sxl<sup>M1</sup> seems to be sensitive to genetic background since the two recombinants gave slightly different suppression at least when combined with specific o<sup>tu</sup> alleles.
Table 2. Enhancement of the ovoD ovarian phenotype by otu mutations

<table>
<thead>
<tr>
<th>Genotype of females</th>
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<th>Statistics</th>
<th>ovoD3</th>
<th>Statistics</th>
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<td>90</td>
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<td>&lt;0.01</td>
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<td>80</td>
<td>0.236</td>
<td>NS</td>
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<td>&lt;0.01</td>
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<td>0.236</td>
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<td>( onc)</td>
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<tr>
<td>otu3 / FM3</td>
<td>0.083</td>
<td>54</td>
<td>0.917</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(qui)</td>
<td>9.240</td>
<td>48</td>
<td>0.344</td>
<td>77</td>
</tr>
<tr>
<td>otu1 / FM7</td>
<td>0.031</td>
<td>48</td>
<td>0.717</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>y cv oto10 v f</td>
<td>3.631</td>
<td>42</td>
<td>11.187</td>
<td>123</td>
</tr>
<tr>
<td>/ FM0 (qui)</td>
<td>3.533</td>
<td>75</td>
<td>15.317</td>
<td>41</td>
</tr>
<tr>
<td>y cv oto14 v f</td>
<td>0.052</td>
<td>86</td>
<td>0.912</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>/ FM3 ( onc)</td>
<td>5.825</td>
<td>57</td>
<td>9.625</td>
<td>44</td>
</tr>
<tr>
<td>y cv oto15 v f</td>
<td>0.000</td>
<td>40</td>
<td>0.000</td>
<td>100</td>
</tr>
<tr>
<td>/ FM7 ( qui)</td>
<td>6.463</td>
<td>43</td>
<td>0.000</td>
<td>100</td>
</tr>
<tr>
<td>otu14 / FM7</td>
<td>6.463</td>
<td>43</td>
<td>0.000</td>
<td>100</td>
</tr>
<tr>
<td>(dif)</td>
<td>0.080</td>
<td>56</td>
<td>2.311</td>
<td>74</td>
</tr>
<tr>
<td>y w otu17 / FM7</td>
<td>0.220</td>
<td>50</td>
<td>3.188</td>
<td>64</td>
</tr>
<tr>
<td>FM7 (qui)</td>
<td>1.500</td>
<td>50</td>
<td>5.581</td>
<td>68</td>
</tr>
<tr>
<td>otuD4 / FM7</td>
<td>0.048</td>
<td>124</td>
<td>0.204</td>
<td>108</td>
</tr>
<tr>
<td>(qui)</td>
<td>2.983</td>
<td>118</td>
<td>9.819</td>
<td>80</td>
</tr>
</tbody>
</table>

Female parents of the indicated genotype were crossed to ovoD2 v24 / Y or ovoD3 v24 f 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 allele 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, SxlM4, 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, ovoD1, ovoD2 and ovoD3, have been isolated (Busson et al., 1983). In heterozygous flies, the strongest allele, ovoD1, 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 ovoD2/ovo+ females, oogenesis usually stops around stage 6 with only a few defective vitellogenic oocytes. The number of vitellogenic ovoD2/ovo+ oocytes depends on the genetic background (Oliver et al., 1990). Although totally sterile, ovoD3/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 ovoD2/ovo+ ovaries are modified in flies that are also heterozygous for some alleles of Sxl or for snf1621 (Oliver et al., 1990).

Thirteen otu alleles, ranging from very strong (quiescent), intermediate (oncogenic, tumorovious), to weak (differentiation-defective; King et al., 1986; Storto and King, 1987; 1988), were tested for dominant interaction with ovoD mutations. Table 2 shows that most alleles strongly interact with either ovoD2 or ovoD3, 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 ovoD and otu (ovoD otu+/otu+ ovo+ otu+) compared to their sibling (ovoD otu+/otu+ ovo+ otu+). No or very few oocytes (1-2 per 10 ovaries with ovoD2 and 1-8 per 10 ovaries with ovoD3) were found in double heterozygotes compared to several oocytes in control flies (4-92 per 10 ovaries with ovoD2 and 55-141 per 10 ovaries with ovoD3). We could not find any obvious correlation between the strength of an otu allele and its interaction with ovoD2 or ovoD3. This is not entirely surprising since these two ovoD mutations are very sensitive to changes of the genetic background (Oliver et al., 1990; D.P., unpublished data): even a
slight reduction of \( \text{o}tu^+ \) activity might be sufficient to give a strong interaction. The only exception was \( \text{o}tu^+ \), which showed no significant interaction with either \( \text{ovo}^{D2} \) or \( \text{ovo}^{D3} \). This allele is the weakest \( \text{o}tu \) mutation so far isolated (King et al., 1986). The \( \text{o}tu^+ \) 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 \( \text{o}tu \) locus is uncovered by two overlapping deletions, \( \text{Df}(1) \text{RA2} \) (7D10;8A4-5) and \( \text{Df}(1) \text{KA14} \) (7F1-2;8C6). They were used to determine whether the interaction observed between \( \text{ovo}^{D2} \) or \( \text{ovo}^{D3} \) and the \( \text{o}tu \) alleles can be obtained simply by changing the dose of \( \text{o}tu^+ \) activity. Table 3A summarizes the interaction observed between \( \text{ovo}^{D2} \) or \( \text{ovo}^{D3} \) and the two deletions uncovering \( \text{o}tu \). With \( \text{Df}(1) \text{RA2} \) and \( \text{ovo}^{D2} \) no vitellogenic oocyte was observed whereas a single egg was found in 89 ovaries of genotype \( \text{ovo}^+ \text{Df}(1) \text{KA14} / \text{ovo}^{D2} \text{Df}^+ \). These numbers are significantly different from those observed in control siblings. A few vitellogenic stages were observed when \( \text{ovo}^{D3} \) was used. These results are in full agreement with those obtained with most \( \text{o}tu \) alleles and are probably due entirely to the dosage of \( \text{o}tu^+ \) activity since none of the mutations that we tested in nine lethal complementation units uncovered by the deletions showed any interaction with \( \text{ovo}^{D2} \) and \( \text{ovo}^{D3} \) (not shown). Moreover, these results indicate that the dominant interaction is dose dependent and does not require the presence of mutant \( \text{o}tu \) proteins.

Dose dependency was further tested by using a parental stock that bears \( \text{Df}(1) \text{RA2} \) and a duplication of the region \( \text{Dp}(1;2) \text{FN107}, 7 \text{A}8 \) 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 \( \text{trans} \) with \( \text{ovo}^{D2} \) and 0.194 with \( \text{ovo}^{D3} \)). In contrast, three copies of the region improved the \( \text{ovo}^{D2} \) and \( \text{ovo}^{D3} \) 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 \( \text{o}tu^+ \) genes (compare \( \text{ovo}^D \text{Df}^{lolo} \) \( \text{Df}(1) \text{RA2} \) in Table 3A with \( \text{ovo}^D \text{Df}^{lolo} \text{Cy}^{O}/+ \) in Table 3B). In summary, one dose of \( \text{o}tu^+ \) enhances the \( \text{ovo}^{D2} \) and \( \text{ovo}^{D3} \) phenotypes whereas three doses acts as suppressor.

The two deletions and the \( \text{o}tu \) alleles were also tested in \( \text{trans} \) with \( \text{ovo}^{D1} \). At first glance, hemizygosity for region 7F-8A or heterozygosity at the \( \text{o}tu \) locus produced little modification of the \( \text{ovo}^{D1} \) ovarian phenotype: comparable numbers of pre-stage 4 egg chambers and no advanced stages were observed in the presence of either one or two \( \text{o}tu^+ \) copies. However, in ovaries with only one wild-type \( \text{o}tu \) 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 \( \text{ovo}^{D1} \) and \( \text{snf}^{1621} \), 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 \( \text{ovo}^D \) alleles and most \( \text{o}tu \) alleles. First, the ovarian

### Table 3. Dependence of \( \text{ovo}^D \) ovarian phenotype on the number of \( \text{o}tu^+ \) genes

(A)

<table>
<thead>
<tr>
<th>Genotype of females</th>
<th>( \text{ovo}^{D2} )</th>
<th>( \text{ovo}^{D3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Mean} )</td>
<td>( \text{No.} )</td>
<td>( \text{Statistics} )</td>
</tr>
<tr>
<td>( \text{Df}(1) \text{RA2} )</td>
<td>( \text{o}vo^{D2}/\text{Df} )</td>
<td>0.000</td>
</tr>
<tr>
<td>( \text{FM7c} )</td>
<td>( \text{o}vo^{D2}/\text{FM7c} )</td>
<td>0.949</td>
</tr>
<tr>
<td>( \text{Df}(1) \text{KA14} )</td>
<td>( \text{o}vo^{D2}/\text{Df} )</td>
<td>0.034</td>
</tr>
<tr>
<td>( \text{FM7c} )</td>
<td>( \text{o}vo^{D2}/\text{FM7c} )</td>
<td>9.890</td>
</tr>
<tr>
<td>( \text{Df}(1) \text{KA14} )</td>
<td>( \text{ovo}^{D2}/\text{Df} )</td>
<td>0.000</td>
</tr>
<tr>
<td>( \text{FM6} )</td>
<td>( \text{ovo}^{D2}/\text{FM6} )</td>
<td>7.710</td>
</tr>
</tbody>
</table>

(B)

| Genotype of females | \( \text{Mean} \) | \( \text{No.} \) | \( \text{Statistics} \) | \( \text{Mean} \) | \( \text{No.} \) | \( \text{Statistics} \) |
|---------------------|-----------------|-----------------|
| \( \text{Parent} \) | \( \text{Progeny} \) | \( \text{Number of} \) | \( \text{o}tu^+ \text{ copies} \) | \( \text{Oocytes/ovary} \) | \( \text{Mean} \) | \( \text{No.} \) | \( \text{Statistics} \) | \( \text{Oocytes/ovary} \) | \( \text{Mean} \) | \( \text{No.} \) | \( \text{Statistics} \) |
| \( \text{Df}(1) \text{RA2}/\text{FM7a} \) | \( \text{o}vo^{D2}/\text{Cy}^{O} \) | 1 | 0.000 | 74 | 0.194 | 85 |
| \( \text{Dp}(1;2) \text{FN107}/\text{Cy}^{O} \) | \( \text{o}vo^{D2}/\text{Df}^+ \) | 2 | 1.610 | 73 | 8.635 | 78 |
| \( \text{o}vo^{D2}/\text{FM7a} \) | \( \text{o}vo^{D2}/\text{Cy}^{O} \) | 2 | 0.700 | 80 | 6.455 | 77 |
| \( \text{o}vo^{D2}/\text{FM7a} \) | \( \text{o}vo^{D2}/\text{Df}^+ \) | 3 | 7.481 | 79 | 12.966 | 74 |

See Table 2 for details. ND, not done. The \( \text{Cy}^{O} \) balancer chromosome was recognized by the dominant wing phenotype \( \text{Cy} \). The differences between one, two or three \( \text{o}tu^+ \) genes are all highly significant.
Fig. 5. Synergistic interaction between *ovo* and *otu* alleles. (A) Ovary with an egg chamber containing germ cells that resemble spermatocytes. Genotype: *ovo* / *ovo* / *otu* / / *otu*. 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* / / *otu* / / *otu*. 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 ovoD2/+ and ovoD3/+ flies are dependent on the dose of the otu* gene. Second, synergistic interaction was found between otu alleles and ovoD1, leading to the sexual transformation of some 2X:2A germ cells towards male differentiation. These interactions are similar to those observed between ovoD 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 (bgen, fes, fu, see Lindsley and Zimm, 1992 for detailed description of these three loci; bam, McKearin and Spradling, 1990; fl/2d, 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. S. 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 snf1621, fu1 or otu1 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/2d 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/2d (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 otu1, otu11 or otu13 whose primary phenotype is tumorous ovaries. The fertility of some heteroallelic combinations (otu/otu11 or otu/otu13) 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, SxlM1 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 snf1621 allele. In flies doubly heterozygous for ovoD1 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 ovoD2 and ovoD3, a clear dosage effect was
observed: decreasing the number of wild-type otu genes enhances the ovoD 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 ovoD2 and ovoD3 ovaries. We would like to stress that the interaction with ovoD2 and ovoD3 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; Tirrnonen 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 otu11, it leads to an amino acid substitution while in otu12 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 fli2d. 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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