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Molecular characterization of ovarian tumors in Drosophila

Eunkyung Bae[a], Kevin R. Cook[a], Pamela K. Geyer[b], Rod N. Nagoshi[a]

[a]*Department of Biological Sciences,*
[b]*Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242-1234, USA*

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Abstract

Certain female-sterile mutations in *Drosophila* result in the uncontrolled proliferation of *X/X* germ cells. It has been proposed that this ovarian tumor phenotype results from the sexual transformation of *X/X* germ cells to a male identity. We present findings inconsistent with this model. We demonstrate that the tumorous cells produced by mutations in the *ovarian tumor* (*otu*), *Sex-lethal* (*Sxl*) and *sans fille* (*snf*) genes are capable of female-specific transcription and RNA processing. This indicates that these ovarian tumor cells still retain some female identity. Therefore, we propose that mutations in these genes do not cause a male transformation of the *X/X* germ line but instead either cause an ambiguous sexual identity or block specific stages of oogenesis. Our findings indicate that while *Sxl* is the master sex determination gene in somatic cells, it appears to play a more subsidiary role in the germ line. Finally, we demonstrate that the germ line function of *Sxl* depends on the activity of a specific OTU isoform.

Keywords: Ovarian tumor; Germline; Oogenesis; *Drosophila*; Sex determination

1. Introduction

Mutations in a number of loci affect various aspects of Drosophila oogenesis. These genes can differ in the sex-specificity of their function and the range of their mutant defects. A subset of these mutations affect both the proliferation and differentiation of *X/X* germ cells, resulting in the formation of grossly abnormal egg cysts known as 'ovarian tumors' (King and Riley, 1982). Ovarian tumor cysts consist of hundreds to thousands of germ line cells surrounded by an unaffected somatic follicle cell layer (King, 1979; King and Riley, 1982). The tumorous germ cells are small, mitotically active and fail to differentiate into nurse cells or oocytes.

Much of our understanding of early oogenesis and tumorous cyst formation comes from the study of three ovarian tumor loci, *Sex-lethal* (*Sxl*), *sans fille* (*snf*) and *ovarian tumor* (*otu*). The *Sxl* gene has both somatic and germ line functions (Maine et al., 1985; Nöthiger and Steinmann-Zwicky, 1985; Cline, 1988; Steinmann-Zwicky, 1988; Salz, 1992). In the soma, *Sxl* is the primary sex determination gene that controls the genetic pathways regulating somatic sexual differentiation (Cline, 1984; Maine et al., 1985). In the germ line, a cell-autonomous *Sxl* function is required for oogenesis. This is demonstrated by the findings that pole cell transplantation of Sxl^- cells into normal female hosts (Schüpbach, 1985; Steinmann-Zwicky et al., 1989) and certain hypomorphic alleles of *Sxl* cause female sterility, characterized by small ovaries containing either tumorous egg cysts or arrested late stage egg chambers (Perrimon et al., 1986; Salz et al., 1987; our data). The *snf* gene also functions in both the soma and germ line (Oliver et al., 1988); it is also known as *fs(1)A1621* (Gans et al., 1975) and *liz* (Steinmann-Zwicky, 1988), and probably acts to regulate *Sxl* expression (Oliver et al. 1988; Steinmann-Zwicky 1988; Salz 1992; Albrecht and Salz, 1993). Females homozygous for snf^{1621}, a female-sterile allele of *snf*, form small ovaries containing tumorous egg cysts similar in phenotype to those resulting from the female-sterile *Sxl* alleles (Perrimon et al. 1986; Salz et al. 1987; Oliver et al. 1988; Steinmann-Zwicky 1988). Lastly, the *otu* gene is required in the

* Corresponding author.

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germ line for proper oogenesis but has no known function in somatic tissue or in males (Wieschaus et al., 1981; Perrimon and Gans, 1983). Alleles of *otu* can produce ovaries of three phenotypic classes; those lacking egg chambers, those containing tumorous egg cysts and those showing late stage arrest of oogenesis (King and Riley, 1982).

Ovarian tumors may result from the disruption of more than one developmental process. For example, mutations in the *lethal(2) giant discs* gene not only affects cell proliferation in imaginal disks, but can also lead to tumorous egg cysts that are morphologically similar to those produced by *otu* mutations (Szabad et al., 1991). In this case, it appears that the misregulation of cell cycle can lead to the abnormal female germ cell proliferation and differentiation associated with ovarian tumors. A related mechanism was proposed to explain the mutant phenotypes of *otu* alleles (King and Storto, 1988). Early oogenesis requires the formation of a 16 germ cell syncytium that is held together by cytoplasmic bridges (King, 1979; King and Storto, 1988). Mutations in *otu* disrupt the formation of these bridges, which may result in the uncontrolled cell division associated with ovarian tumors (King, 1979).

Both these hypotheses suggest that tumorous egg cysts result from mutations in functions required for various aspects of cell division. In contrast, an alternative model proposes that the ovarian tumor phenotype results from the aberrant sexual differentiation of *X/X* germ cells. In this model, the ovarian tumor genes are proposed to be required for female germ line differentiation. Mutations in these genes cause *X/X* germ cells to be transformed to a male identity, leading to a sexual incompatibility between the mutant germ line and the surrounding somatic ovary. This disrupts the regulation of cell proliferation, thereby causing the formation of ovarian tumors (Steinmann-Zwicky et al., 1989; Pauli and Mahowald, 1990). Support for this model comes from several observations including: (1) Tumorous cysts can form in females with intermediate X:A ratios, i.e. 2X:3A intersexes (Schüpbach, 1985). (2) Cysts similar to ovarian tumors form when *X/Y* pole cells are transplanted into a female host (Schüpbach, 1985; Steinmann-Zwicky et al., 1989). (3) *X/X* cells of ovarian tumor cysts are morphologically similar to primary spermatocytes, suggesting a male germ cell identity (Oliver et al., 1988, 1990; Steinmann-Zwicky, 1992). (4) Some male specific products are expressed in ovarian tumors, including a male-specific RNA splice variant of *Sxl* (Oliver et al., 1993; Bopp et al., 1993), the expression of spermatogenesis specific enhancer traps (Pauli et al., 1993; Wei et al., 1994) and certain male-specific RNAs (Wei et al., 1994). Observations (1) and (2) demonstrate that an ovarian tumor phenotype can result from an incompatibility between the chromosomal sex of the germ cells and the soma.

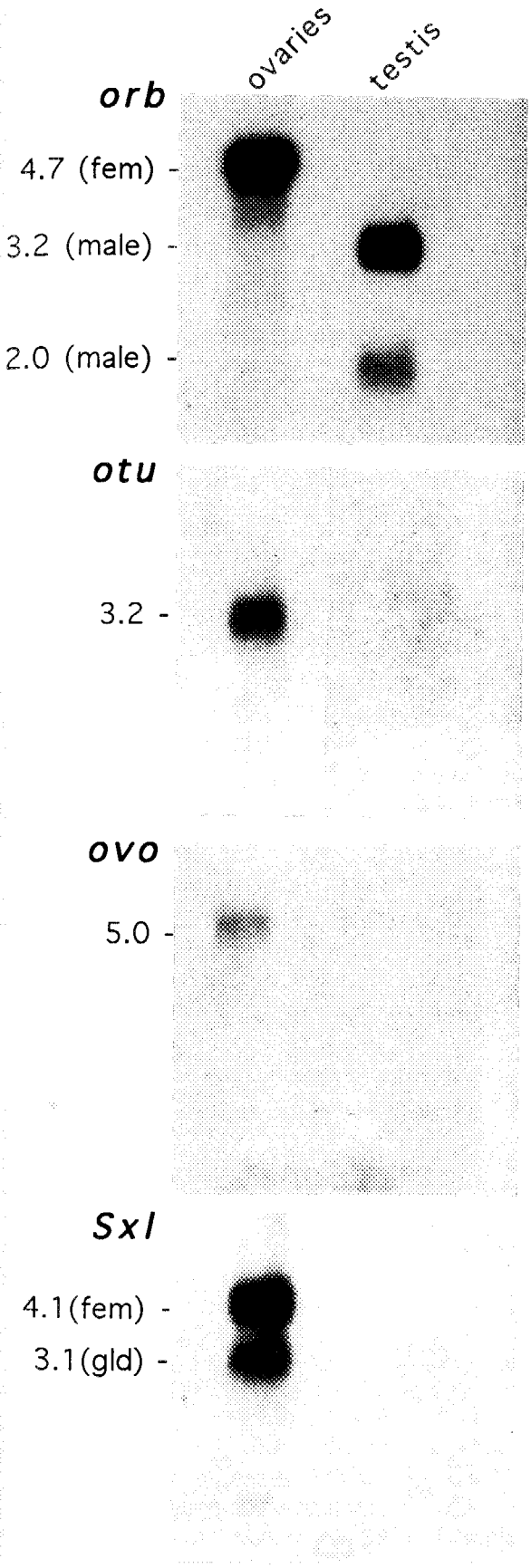
We are interested in defining the role that *otu* plays in oogenesis. This includes understanding how *otu* mutations cause ovarian tumors and determining the possible regulatory relationships between *otu* and the other ovarian tumor genes. We first examined whether the *otu* gene is required to establish female identity in the germ line, as has been recently proposed (Oliver et al., 1993; Pauli et al., 1993; Wei et al., 1994). We reasoned that if *otu* mutations act to transform the *X/X* germ line to a male identity, this should lead not only to ectopic expression of male products but also the repression of genes required for oogenesis. These studies were extended to examine expression patterns of female-specific genes in morphologically similar ovarian tumors produced by *snf* and *Sxl* mutations. We found that our results were relevant to recent studies suggesting that *otu* acts upstream of *Sxl* in a genetic pathway (Pauli et al., 1993). We therefore examined in more detail the relationship between *otu* and *Sxl* in oogenesis by correlating *Sxl* expression with the activity of specific OTU isoforms.

2. Results

2.1. Determining the differentiation state of ovarian tumor cells by using sex-specific molecular markers

Mutations in *Sxl*, *otu* and *snf* result in female sterility characterized by the formation of ovarian tumors. Previous experiments have shown that these tumorous cells express male specific products and lead to the suggestion that they represent a sexual transformation of female germ cells to a male (spermatogenic) developmental fate (Oliver et al., 1993; Pauli et al., 1993). To test this proposal, we examined whether these tumorous cells still retained some female identity by monitoring the expression of genes required for oogenesis. Specifically, we studied a subset of the female germ line-dependent RNAs produced from the *Sxl* (Bell et al., 1988; Bopp et al., 1991; Samuels et al., 1991), *otu* (Mulligan et al., 1988; Comer et al., 1992; Geyer et al., 1993), *orb* (Lantz et al., 1992) and *ovo* (Mevel-Ninio et al., 1991; Garfinkel et al., 1992) loci. In each case, total RNA was isolated from dissected ovaries and examined by Northern analysis. Because these genes are required for oogenesis, their expression is not only useful as molecular markers for female germ line identity but is also likely to have functional consequences to the differentiation of the germ cell.

Because the mutations tested have gross effects on the morphology of germ cells, it would not be surprising if they nonspecifically disrupted gene regulation, altered the proportion of germ cells to soma in the ovary or gave rise to ovaries containing egg cysts of variable mutant morphologies and developmental ages. Any of these conditions could non-specifically change the relative levels of the germ line-specific transcripts. For these reasons, we focused on the presence or absence of the



RNAs tested and discounted quantitative variations in their relative amounts.

2.2. Female germ line-dependent ovo, orb and otu expression occurs in tumorous ovaries

We first examined the levels of the female-specific *orb* transcript in ovaries mutant for female-sterile alleles of *otu*, *Sxl* or *snf*. The *orb* gene encodes ovarian- and testis-specific transcripts that differ due to the use of alternative promoters and sex-specific RNA processing (Lantz et al., 1992). The ovarian-specific transcript is first detected early in oogenesis, when cystoblasts are present at the 16 cell stage, and continues to be expressed throughout the development of the egg cysts (Lantz et al., 1992). It is not detected in adult males or in isolated testes (Fig. 1; Lantz et al., 1992). The testis-specific RNA is also expressed at the 16 cell stage, when the male germ line has formed primary spermatocytes. It is detectable through most subsequent stages of spermatogenesis, but is never seen in ovaries (Fig. 1; Lantz et al., 1992). Because of its sex-specificity and expression throughout most of gametogenesis, the *orb* RNAs are useful markers for examining alterations in sex-specific RNA splicing and gene expression in the germ line.

We found that the female-specific *orb* 4.7-kb transcript is present at near wild-type levels in ovaries mutant for *Sxl*, *snf* and *otu* (Fig. 2A). These results indicate that despite the severity of the mutant phenotype, the tumorous germ cells have undergone sufficient female differentiation to promote female-specific promoter activity and RNA processing. It is significant that no male-specific *orb* product was detected (Fig. 2A). This indicates that the tumorous egg cysts do not contain substantial numbers of cells with sufficient male identity for the male-specific regulation of *orb* expression.

Fig. 1. Demonstration that transcripts from *Sxl*, *otu*, *ovo* and *orb* are predominantly expressed in the ovary. Each Northern blot contains 20 μg of total RNA from isolated wild-type ovaries and isolated wild-type testes. The *orb* probe was a 2.1-kb *Eco*RI fragment that will detect one ovarian (4.7 kb — 'fem') and two testis-specific (3.2 kb and 2.0 kb — 'male') RNAs (Lantz et al., 1992). The *ovo*-specific probe was derived from a 0.7-kb *Xho*I fragment that contains sequences from exon 3 and exon 4 (Mevel-Ninio et al., 1991; Garfinkel et al., 1992). This probe fragment does not contain *ovo* sequences believed to be homologous to a repetitive sequence (Garfinkel et al., 1992). The *otu* transcript was detected with a 2.0-kb cDNA probe (Geyer et al., 1993). The *Sxl* probe was derived from a 3.4-kb genomic fragment that detects a 4.1-kb ('fem') female-specific transcript and the 3.1-kb female germ line-dependent ('gld') RNA (Probe 'j' from Salz et al., 1989). This probe should also detect a 4.3-kb male-specific transcript which is not seen. This transcript may not accumulate in isolated testes. To confirm that intact testes RNA was present, each filter was stripped of signal and rehybridized with the *orb* probe. In each case, the testis- and ovarian-specific *orb* transcripts were present at levels similar to that shown in the first filter (data not shown).

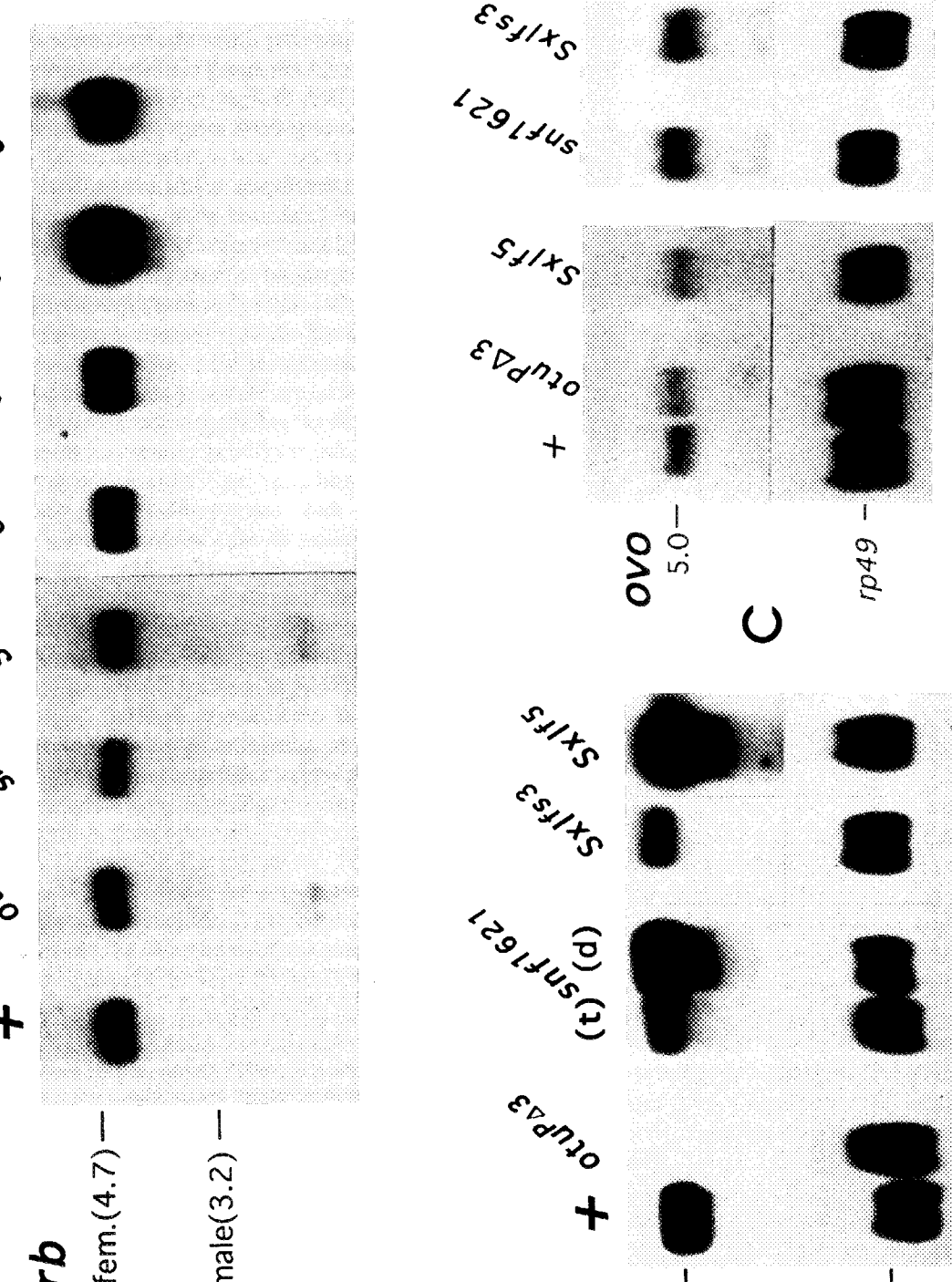


Fig. 2. Northern blot analyses of the female germ line-specific RNAs from *orb*, *otu* and *ovo* in different tumorous ovaries. Mutant allele designation represents ovaries from homozygotes of that allele. Twenty micrograms of total RNA from isolated ovaries were loaded in each lane. (A) The filters were hybridized to *orb* probe described in Fig. 1. The arrow points to the predicted location of the major testis-specific transcript. (B) Hybridization was done with an *otu*-specific probe as in Fig. 1. Symbols: +, wild-type; (t), tumorous ovaries from snf^{1621} mutant mothers; (d), ovaries containing differentiated egg cysts from snf^{1621} mutant mothers. These cysts are arrested late in oogenesis and contain nurse cells and oocytes. (C) Northern blots hybridized with a probe specific to *ovo* as described in Fig. 1. These and subsequent Northerns blots were hybridized to a genomic *rp49* probe as a control for the amount of RNA loaded (O'Connell and Rosbash, 1988).

We next examined the level of accumulation of the *ovo* female germ-line transcript. Mutations in *ovo* result in female sterility characterized by the loss of germ cells (Oliver et al., 1987; 1990). The *ovo* locus is associated with a 5.0-kb RNA that is greatly enriched in the female germ line (Mevel-Ninio et al., 1991; Garfinkel et al., 1992). However, this transcript is not detected by Northern analysis of testes RNA, even under hybridization conditions that readily detect the *orb* testis-specific transcript (Fig. 1). The *ovo* 5.0-kb transcript is found in all tumorous ovaries tested, indicating that the activity of the *ovo* promoter is not significantly altered by reductions in the levels of *Sxl*, *snf*, or *otu* products (Fig. 2C).

The accumulation of the *otu* transcript was examined in *Sxl*, snf^{1621} and *otu* mutant backgrounds by Northern analyses. Probes from the *otu* locus detect a 3.2-kb transcript from adult ovary RNA (Mulligan et al., 1988; Comer et al., 1992; Geyer et al., 1993). This transcript is expressed in the female germ line and is not detectable by Northern analysis in isolated testes (Fig. 1). We found that like the ovarian *orb* RNA, the *otu* RNA is present in ovaries mutant for two female-sterile alleles of *Sxl* (Sxl^{fs3} and Sxl^{f5}) that produce tumorous egg cysts when homozygous (Fig. 2B).

We separately examined severe and more mildly affected snf^{1621} mutant ovaries because of variability in the mutant phenotype. *otu* RNA was present in both mutant classes. However, the tumorous ovaries (t) had reduced levels of the *otu* RNA compared to genotypically identical ovaries with more mature egg chambers (d), indicating a possible correlation between the amount of *otu* transcript and the severity of the mutant phenotype (Fig. 2B). This could result from either differences in the amount of germ line tissue present or variations in the level of *otu* gene expression within germ cells. In either case, it is clear that the *otu* transcript accumulates in ovaries of snf^{1621} females, even when the egg cysts are completely tumorous.

2.3. A female germ line-dependent Sxl transcript is absent in tumorous ovaries

A different result was seen in our examination of a female germ line-dependent *Sxl* transcript. Multiple *Sxl* sex-specific RNAs have been identified that differ by alternative RNA splicing, transcription start sites and transcription termination (Bell et al., 1988; Salz et al., 1989; Bopp et al., 1991; Samuels et al., 1991). A 3.1-kb transcript is expressed in ovaries and is absent in agametic females, whole males and isolated testes (Salz et al., 1989; Samuels et al., 1991). Despite its female germ line specificity, the 3.1-kb RNA probably does not encode the only germ line SXL product that accumulates early in oogenesis. Mutants that lack this transcript can still produce detectable levels of SXL protein in germarial cells (Bopp et al., 1993). Completely overlapping the 3.1-kb RNA is a 4.1-kb transcript that is expressed in somatic tissue and in the female germ line (Salz et al., 1989; Samuels et al., 1991). The 4.1-kb and 3.1-kb transcripts have identical open reading frames that contain the consensus RNA binding domains required for the regulation of sex-specific splicing by *Sxl*. Although the function of the 3.1-kb RNA is not known, its sex-specific and germ line dependent expression pattern make it a useful marker of female germ line differentiation.

To confirm the sex-specificity of the 3.1-kb *Sxl* transcript, we hybridized total RNA from isolated ovaries and testes with a probe that recognizes both the 3.1-kb and 4.1-kb *Sxl* RNAs, as well as a male-specific 4.3-kb transcript (Fig. 1; probe 'j' from Samuels et al., 1991). The 3.1-kb and 4.1-kb transcripts were only present in the ovarian RNA. Curiously we did not see the 4.3-kb male specific RNA in testes, even under conditions that detected the male *orb* transcript. The 4.3-kb *Sxl* transcript is present in relatively low levels in whole adult males and it may be that it does not readily accumulate in testes (Salz et al., 1989; Samuels et al., 1991).

In ovaries mutant for snf^{1621}, Sxl^{fs3} or more severe tumorous *otu* alleles ($otu^{P\Delta 3}$, otu^{1}, otu^{13}), the 3.1-kb *Sxl* RNA was absent while the 4.1-kb *Sxl* transcript accumulated at substantial levels (Fig. 3A). These results indicate that *otu* and *snf* may act before the expression of the 3.1-kb germ line-dependent transcript. Furthermore, the absence of detectable levels of this RNA in the mutant ovaries demonstrates that few, if any, germ cells are present that have progressed to a stage in oogenesis when this transcript is expressed. Apparently, the severe tumorous alleles of *otu*, *Sxl* and *snf* block the development of the great majority of the germ cells at an early stage.

Ovaries mutant for less severe *otu* alleles (otu^{3} and otu^{11}) expressed detectable levels of the 3.1-kb *Sxl* RNA. While otu^{3} and otu^{11} mutant ovaries consist primarily of tumorous egg cysts, approximately 20% progress to later stages of oogenic development (King et al., 1986). However, most of the late stage cysts lack yolk deposition and do not contain fully developed egg chambers (King et al., 1986). This suggests that the 3.1-kb RNA is first expressed about the time of nurse cell differentiation and prior to the most mature stages in oogenesis.

2.4. The Sxl product can partially substitute for the absence of one OTU isoform

The finding that *otu* mutations can affect the expression of a *Sxl* transcript in the germ line led to the examination of how the *Sxl* and *otu* genes interact. Recently, it was shown that a constitutive allele of *Sxl*, Sxl^{M1}, can partially suppress the affects of *otu* mutations by allowing the development of later oogenic stages (Pauli et al., 1993). However, this epistatic interaction was

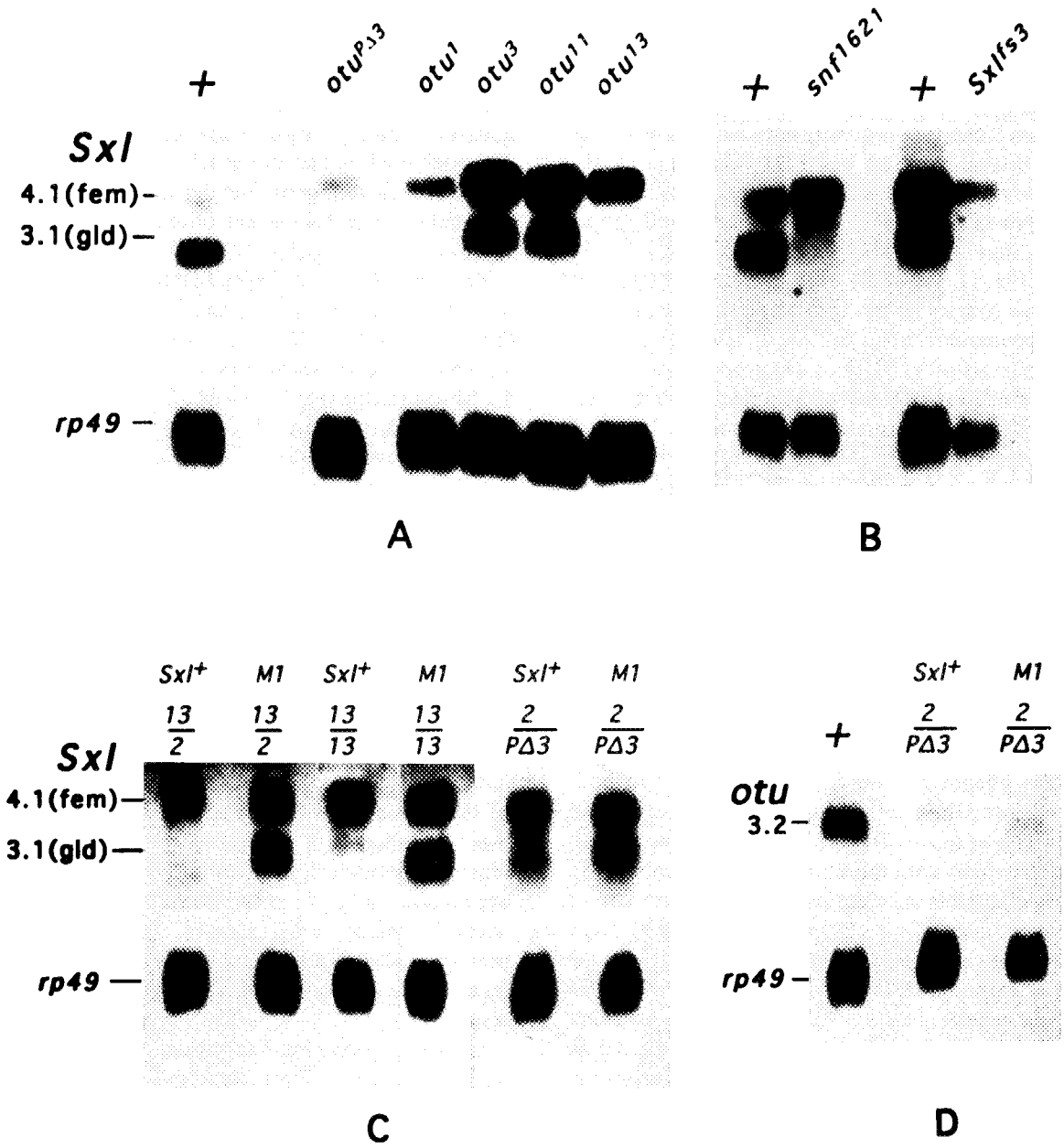


Fig. 3. Northern blot analyses of the female germ line-specific RNAs from *Sxl* and *otu* in different tumorous ovaries. Twenty micrograms of total RNA from isolated ovaries were loaded in each lane. (A and B) Mutant allele designation represents ovaries from homozygotes of that allele. (A, B and C) The filters were hybridized with the *Sxl*-specific probe described in Fig. 1 that detects the 4.1-kb female-specific transcript (fem) and the 3.1-kb female germ line-dependent RNA (gld) (Probe 'j' from Salz et al., 1989). (D) The filters were hybridized to the 2.0-kb cDNA probe specific for the *otu* transcript as described in Fig. 1 (Geyer et al., 1993). Symbols: Sxl^+, homozygous for the wild-type *Sxl* allele; *M1*, Sxl^{M1}/Sxl^+; *13/2*, otu^{13}/otu^2; *13/13*; homozygous for otu^{13}; *2/PΔ3*,$otu^2/otu^{PΔ3}$; +, wild-type for *Sxl*, *snf* and *otu*.

allele-specific and variable. Even in cases where phenotypic suppression occurred the females generally remained sterile. In the over 40 different *otu* allele combinations tested only one, otu^1/otu^{13}, could be suppressed from complete sterility to partial fertility by Sxl^{M1} (Pauli et al., 1993).

We wondered whether this allele specificity was related to the fact that the *otu* gene produces two polypeptides of 98 kD and 104 kD (Steinhauer et al., 1989; Steinhauer and Kalfayan, 1992). It is possible that the degree of suppression by Sxl^{M1} depends on which OTU isoform is affected by the particular *otu* allele combination examined. The 104-kD OTU product differs from the 98-kD isoform by the inclusion of an additional

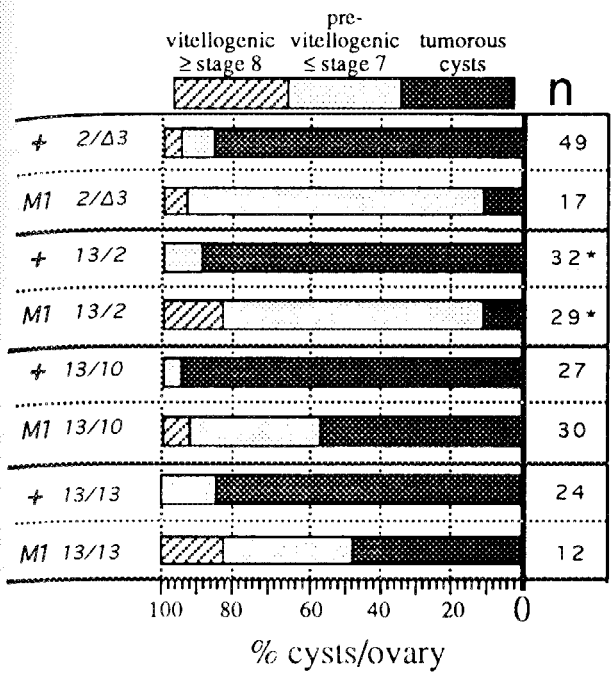


Fig. 4. The phenotypic suppression of *otu* mutations by constitutive *Sxl* alleles. The ovarian phenotypes of *otu* allele combinations were examined with (MI) and without (+) Sxl^{M1}. The n column indicates the total number of ovaries examined per experiment. Tumorous cysts were defined as egg chambers containing many (>100) small, undifferentiated cells. In some *otu-Sxl* combinations, tumorous cysts contained nurse cells and what appeared to be degenerating polyploid nuclei. The pre-vitellogenic and vitellogenic egg chambers were staged as described by King (1970). (*) indicate that the results of two separate experiments have been pooled. (A) The percent of egg cysts per ovary of a particular phenotypic class. Nos. indicate the *otu* allele combination tested (i.e., *2/1* = otu^2/otu^1). Δ3 represents $otu^{PΔ3}$.

Table 1
Mutant alleles used in this study

Allele	Phenotype[a]	Reference
otu^1	Tumorous ovaries	Lindsley and Zimm, 1992
otu^2	Chamberless ovaries	Lindsey and Zimm, 1992
otu^3	Tumorous ovaries	Lindsey and Zimm, 1992
otu^7	Late stage arrest of oogenesis	Lindsley and Zimm, 1992
otu^{10}	Chamberless ovaries	Lindsley and Zimm, 1992
otu^{11}	Tumorous ovaries	Lindsley and Zimm, 1992
otu^{13}	Tumorous ovaries	Lindsley and Zimm, 1992
otu^{14}	Late stage arrest of oogenesis	Lindsley and Zimm, 1992
$otu^{PΔ3}$	Tumorous ovaries	Steinhauer and Kalfayan, 1992
Sxl^{M1}	Dominant, constitutive	Lindsey and Zimm, 1992
Sxl^{f3}	Tumorous ovaries	Oliver et al., 1990
Sxl^{f4}	Tumorous ovaries	Lindsey and Zimm, 1992
Sxl^{f5}	Tumorous ovaries	Lindsley and Zimm, 1992
snf	Tumorous ovaries	Oliver et al., 1988

[a]The average mutant phenotype for loss of function *otu*, *Sxl* and *snf* alleles.

exon encoding 42 amino acids (Steinhauer and Kalfayan, 1992). The use of lesions that map to this exon allowed us to test the effect of Sxl^{M1} on *otu* mutations that specifically interfere with the expression or function of the 104-kD isoform. Unfortunately, we could not do the reciprocal experiment, as any lesion that disrupts the coding region for the 98-kD isoform also affects the 104-kD sequence.

We examined flies specifically defective for the 104-kD product. The otu^{13} lesion disrupts the splice acceptor site of the exon specific for the 104-kD isoform such that only the 98-kD product is made (Steinhauer and Kalfayan, 1992). Therefore, otu^{13} homozygotes are unable to produce any 104-kD product but should express diploid levels of the 98-kD isoform. In a Sxl^+ background, otu^{13}/otu^{13} ovaries contained predominantly tumorous egg cysts (Fig. 4; King et al., 1986). This observation indicates that the 98-kD OTU isoform is sufficient to allow *X/X* germ cell proliferation, but cannot support their differentiation into nurse cells or oocytes in the absence of the 104-kD OTU product. We found that the addition of Sxl^{M1} not only suppressed the otu^{13} mutant phenotype by increasing the frequency of more mature egg cysts (Fig. 4), but was able to completely rescue some egg cysts such that partial fertility and fecundity were obtained (Table 2). This indicates that the constitutive expression of *Sxl* can completely substitute for the absence of the 104-kD OTU isoform.

This observation was supported by experiments with the allele combination, otu^{13}/otu^{11}. The otu^{11} allele is a single missense mutation in the exon specific for the 104-kD isoform (Steinhauer and Kalfayan, 1992). Therefore, like otu^{13}, this allele should be defective for the 104-kD product but still able to produce normal levels of the 98-kD isoform. otu^{13}/otu^{11} females in a Sxl^+ background are completely sterile, producing small ovaries containing mostly tumorous egg cysts (Table 2; King et al., 1986). With the addition of Sxl^{M1}, 100% of otu^{13}/otu^{11} females were fertile and had high fecundity (Table 2). The dramatic difference in the degree of suppression in otu^{13}/otu^{11} flies compared to otu^{13}/otu^{13} homozygotes is probably because the otu^{11} allele retains some 104-kD activity. This is supported by the findings that otu^{11} can partially complement some *otu* alleles that otu^{13} cannot (Storto and King, 1987), and that otu^{11}/otu^{11} flies produce detectable levels of an immuno-reactive 104-kD product (Steinhauer and Kalfayan, 1992). The enhanced suppression resulting from additional 104-kD function may indicate that Sxl^{M1} is not completely constitutive in the germ line, or that the 104-kD product has an additional Sxl-independent function late in oogenesis.

Our experiments indicate that the 98-kD OTU product is also needed for oogenesis but differs from the 104-kD OTU isoform in that it appears to act independent of *Sxl*. The otu^{10} and otu^2 alleles are severe *otu* muta-

Table 2
Fertility and fecundity of *otu* mutations suppressed by Sxl^{M1}

Genotype	n[a]	Est. level of 98 kD OTU	Est. level of 104 kD OTU	No. of fertile females (%)[b]	Average no. of progeny[c]	Range (low-high)[d]
Sxl^+ otu^{13}/Sxl^+ otu^+	49	Normal	Normal	49 (100)	>50	All >50
Sxl^+ otu^{13}/Sxl^+ otu^{13}	53	Normal[e]	None[e]	0 (0)	0	—
Sxl^{M1} otu^{13}/Sxl^+ otu^{13}	20	Normal[e]	None[e]	11 (55)	7.8	1–20
Sxl^+ otu^{13}/Sxl^+ otu^{11}	48	Normal[e]	Low[e]	0 (0)	0	—
Sxl^{M1} otu^{13}/Sxl^+ otu^{11}	41	Normal[e]	Low[e]	41 (100)	>50	All >50
Sxl^{M1} otu^{13}/Sxl^+ otu^2	30	Moderate	None-low	0 (0)	0	—
Sxl^{M1} otu^{13}/Sxl^+ otu^{10}		Moderate	None-low	0 (0)	0	—
Sxl^{M1} otu^2/Sxl^+ otu^7	28	Moderate[f]	Moderate[f]	0 (0)	0	—
Sxl^{M1} otu^2/Sxl^+ otu^{14}	31	Moderate[f]	Moderate[f]	0 (0)	0	—

[a]Total number of females examined for each genotype.
[b]Individual females were mated with three males for 14 days at 25°C and examined for progeny.
[c]Number of progeny per female after 14 days at 25°C.
[d]Low and high extremes of no. of progeny from fecundity test.
[e]Based on molecular characterization of the mutation (Steinhauer and Kalfayan, 1992).
[f]Based on weak mutant phenotype in Sxl^+ background (Storto and King, 1987).

tions that probably eliminate the function of both OTU isoforms. Therefore, otu^{13}/otu^2 and otu^{13}/otu^{10} mutant flies are mostly, if not entirely, deficient for the 104-kD product but should be able to make half the diploid level of the active 98-kD isoform. In a Sxl^+ background, otu^{13}/otu^2 and otu^{13}/otu^{10} ovaries contained predominantly tumorous egg cysts (Fig. 4; King et al., 1986). When Sxl^{M1} was added, only incomplete suppression of the mutant phenotype was seen, as Sxl^{M1} otu^{13}/Sxl^+ otu^2 and Sxl^{M1} otu^{13}/Sxl^+ otu^{10} females contained ovaries with more advanced stages of oogenesis but remained sterile (Table 2, Fig. 4). This inability of otu^{13}/otu^{10} or otu^{13}/otu^2 flies to be suppressed to fertility, like otu^{13} homozygotes, must be due to insufficient 98-kD function. Apparently, the constitutive expression of *Sxl* will allow the differentiation of nurse cells and oocytes in the absence of the 104-kD OTU product, but is not sufficient to support the maturation of functional egg chambers when the activity of the 98-kD OTU isoform is reduced.

Our results delineate separate functions and periods of activity for the two OTU isoforms. The 98-kD OTU isoform is sufficient to allow proliferation of *X/X* germ cells during early oogenesis and is also involved in later stages of oogenesis. The 104-kD OTU isoform is required for the differentiation of nurse cells and oocytes by some mechanism that involves the germ line *Sxl* function. Mutations in either the 104-kD product or *Sxl* result in the formation of ovarian tumors. How *otu* and *Sxl* interact is not clear. There are at least three mechanisms compatible with the genetic epistasis results: (1) the *otu* gene acts upstream of *Sxl* and is required for *Sxl* activity, as has been previously suggested (Pauli et al., 1993); (2) the *Sxl* product and the OTU 104-kD isoform have parallel and partially overlapping activities so that the overexpression of *Sxl* by the Sxl^{M1} allele can compensate for reduced levels of the OTU product; (3) *Sxl* is required for *otu* activity such that the overexpression of *Sxl* increases the expression of the hypomorphic *otu* alleles, thereby allowing the development of later oogenic stages.

2.5. Molecular evidence that Sxl germ line activity does not regulate otu expression

To better understand the order of *otu-Sxl* action in oogenesis, we performed molecular epistasis experiments in which the levels of the *otu* and *Sxl* RNAs in suppressed and unsuppressed *otu* mutant ovaries were examined. We reasoned that if *Sxl* regulates *otu*, then the suppression of *otu* mutants by the constitutive expression of *Sxl* should correlate with an increase in *otu* RNA production. Alternatively, if *Sxl* suppression does not affect *otu* RNA levels, it would indicate that *otu* acts prior to, or in parallel with, *Sxl* in the germ line.

Flies of the genotype $otu^2/otu^{P\Delta 3}$ have small tumorous ovaries that lack detectable levels of the *Sxl* germ line transcript (Fig. 3C) and have greatly reduced amounts of *otu* RNA (Fig. 3D). As expected, females that contain the constitutive Sxl^{M1} allele ($Sxl^{M1}otu^2/Sxl^+$ $otu^{P\Delta 3}$) contain ovaries with a dramatically improved morphology, (Fig. 4). The majority of the suppressed egg cysts contained nurse cells and a significant fraction had detectable yolk deposition. We demonstrated by Northern analysis that this phenotypic improvement did not result from an increase in *otu* gene expression (Fig. 3D). These data are inconsistent with a model in which Sxl^{M1} acts to increase activation of the hypomorphic *otu* alleles. Therefore, *otu* must act upstream or in parallel with *Sxl*.

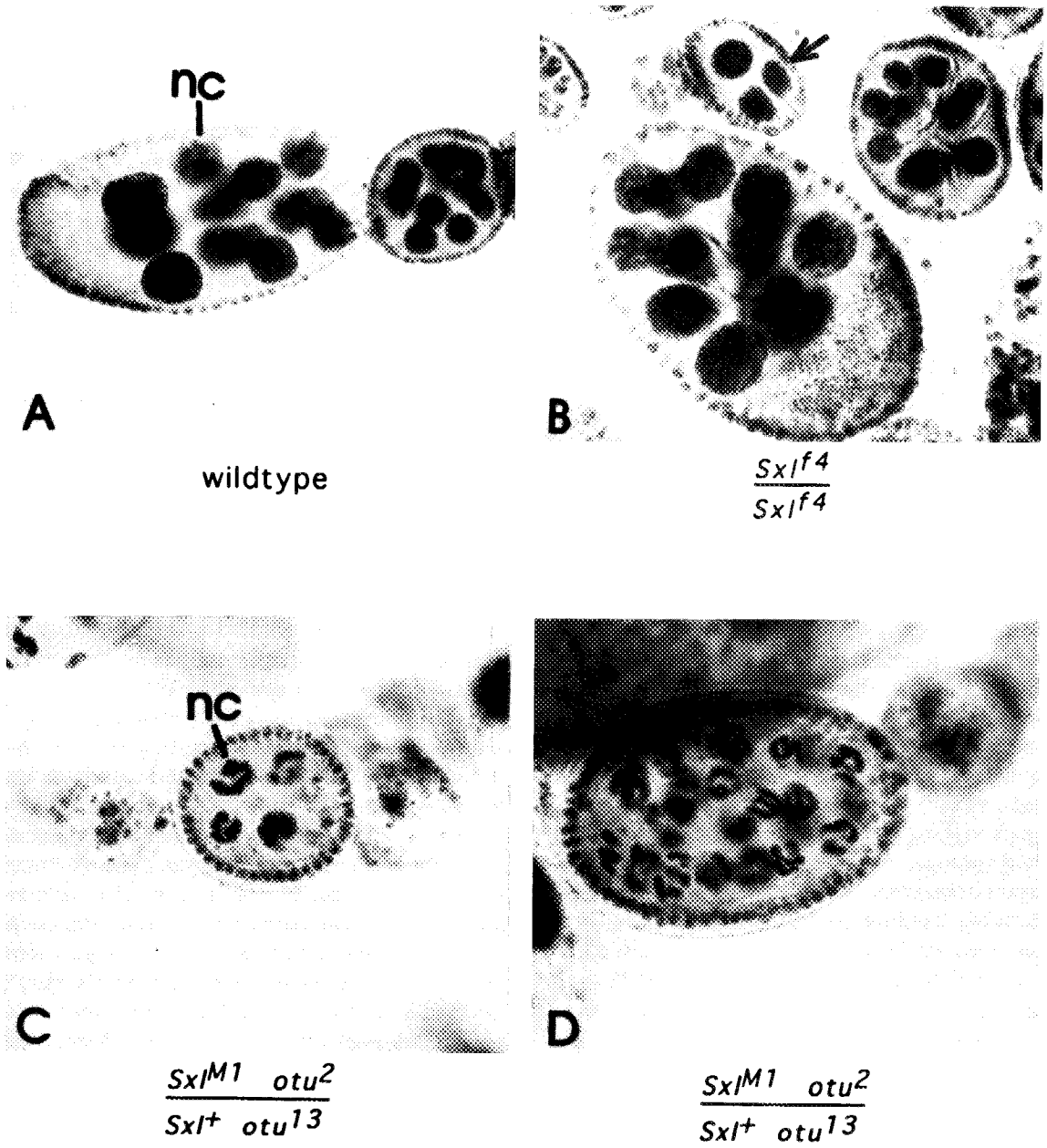


Fig. 5. The chromosome morphology and the number of nurse cells per egg chamber are affected by *otu* mutations and cannot be suppressed by Sxl^{M1}. Wild-type egg cysts contain 15 polyploid nurse cells (nc). Chromosomes are stained by Feulgen reagent. All cysts are shown under bright field microscopy using a 40× objective. Arrow indicates cyst with three nurse cells. Note the polytene chromosomes present in *otu* mutant cysts (C and D).

The suppression in the *otu* mutant phenotype by constitutive *Sxl* activity occurred concomitant with an increase in the levels of the *Sxl* 3.1-kb germ line transcript (Fig. 3C). otu^{13}/otu^{2}, otu^{13}/otu^{13} and $otu^{2}/otu^{P\Delta 3}$ produce tumorous ovaries that express reduced levels of the 3.1-kb RNA. The addition of Sxl^{M1} resulted in a dramatic increase in the expression of this transcript without affecting the levels of the *Sxl* 4.1-kb RNA. These results demonstrate that the expression of the *Sxl* 3.1-kb RNA is specifically influenced by the level of *otu* activity, either by affecting the regulation of *Sxl* expression or by allowing progression to those later stages of oogenesis when the 3.1-kb transcript is produced.

2.6. A Sxl-independent function of otu is required for late stages of oogenesis, including maturation of the egg chamber, nurse cell polyteny and nurse cell number

We further extended the analysis of Pauli et al. (1993) to examine whether Sxl^{M1} could suppress weak *otu* alleles that allow substantial oogenic development. Two *otu* allele combinations, otu^2/otu^7 and otu^2/otu^{14}, produce ovaries containing predominantly late staged egg cysts that are often vitellogenic (King and Riley, 1982). Even though ovaries in this phenotypic class produce more *otu* product than tumorous ovaries (Geyer et al., 1993; Sass et al., 1993), neither of these *otu* mutants were suppressed by Sxl^{M1} (Table 2). Sxl^{M1} otu^2/Sxl^+ otu^7 and Sxl^{M1} otu^2/Sxl^+ otu^{14} females remained sterile and there was no improvement in the morphology of the ovaries.

Other abnormalities associated with the late stage arrest of oogenesis by *otu* mutations are not suppressed by constitutive *Sxl* expression. One such phenotype is the formation of nurse cells containing polytene chromosomes. Wild-type nurse cells normally contain large, polyploid nuclei resulting from 7–8 rounds of endoreplication of the nurse cell genome (Mulligan and Rasch, 1985). Initially, the homologous chromosomes are in register but they quickly dissociate, resulting in a fibrous mass of oligotene threads that are distributed throughout the nucleus (King et al., 1981). This causes a dispersed and punctated nuclear staining pattern with DNA-specific dyes (Fig. 5A). In ovaries resulting from some *otu* mutations, the multiply replicated chromosomes remain in register to form condensed strands that have detectable banding patterns (Dabbs and King, 1980; King et al., 1981). We found that Sxl^{M1} could not suppress this polytene phenotype (Fig. 5C and D).

A second phenotype characteristic of *otu* mutations is that they frequently produce egg chambers with fewer than the expected 15 nurse cells per cyst. These chambers are smaller and less developed than those with the normal nurse cell number (Fig. 5C). Less frequently, cysts with greater than 15 nurse cells are present. The variation in the number of nurse cells is quite large, ranging from cysts holding as few as one nurse cell to others containing as many as 30. The addition of Sxl^{M1} in *otu* mutants did not suppress this abnormality in nurse cell numbers (Fig. 5C and D).

3. Discussion

3.1. What is the sex of ovarian tumor cells?

Determining the sexual identity of the tumorous germ cell is central to our understanding of the germ line functions of *Sxl*, *snf*, *otu* and germane to the question of how ovarian tumors form. Previous experiments have shown that male-specific splicing of *Sxl* and the activation of certain male-specific promoters occurs in ovarian tumors (Bopp et al., 1993; Oliver et al., 1993; Pauli et al., 1993). These observations led to the proposal that the ovarian tumors produced by mutations in *Sxl*, *snf* and *otu* result from the sexual transformation of *X/X* germ cells to a male identity (Steinmann-Zwicky et al., 1989; Oliver et al., 1993; Pauli et al., 1993). If true, then this switch from a female to a male developmental pathway should not only result in the ectopic expression of male-specific genes, but should also block the activation of genes required for oogenesis. We tested this model by examining the expression of female germ line-dependent RNAs in ovarian tumors resulting from *otu*, *snf* or *Sxl* mutations. In each case, although the severity of the mutant phenotypes are sufficient to eliminate a *Sxl* germ line-dependent RNA, the tumorous ovaries still express female-specific transcripts from the *ovo*, *otu* and *orb* genes at levels approaching that found in wild type ovaries. Not only are the female-specific promoters of *otu* and *ovo* still active, but the tumorous cells have sufficient female identity to promote female-specific RNA splicing and prevent male-specific processing of the *orb* pre-mRNA.

These results are particularly compelling because the genes analyzed are required for egg development. Their expression in tumorous cells is therefore likely to have functional significance, strongly suggesting that those cells are still oogenic. This contrasts with the male-specific products that have been reported in ovarian tumors. For example, the male specific *Sxl* splice variant does not encode an active product and the functions of the loci identified by the male-specific enhancer trap lines are unknown. While the presence of these products clearly indicate that there is some misregulation of gene expression, they do not preclude the possibility that the tumorous cells still retain female identity.

The observation that both male and female products are expressed in tumorous ovaries can still be reconciled with the model that all tumorous cells are male if the mutant egg cysts are of sexually mosaic composition. If this is true, then only a portion of the germ cells in an ovarian tumor are transformed to a male state by the ovarian tumor mutations, the rest remain female. We believe that this explanation is unlikely for several reasons. First, virtually all the cells in a tumorous egg chamber resulting from *otu* mutations ectopically express the male-specific *Sxl* RNA, suggesting that these cells are similarly affected (Oliver et al., 1993). Similarly, we show that the *orb* transcript is only present in the female splice form in the ovarian tumors resulting from *Sxl*, *otu* and *snf* mutations (Fig. 2A). The *orb* testis RNA cannot be detected, as would have been expected if a substantial number of male germ cells were present in the tumorous cysts. In addition, ovarian tumors do not express the female specific 3.1-kb *Sxl* RNA (Fig. 3A and B). This indicates that few, if any, of the mutant *X/X* germ cells proceed to late stages of oogenesis. These re-

sults demonstrate that the tumorous ovaries are composed predominantly of similarly affected germ cells.

3.2. Ovarian tumors are sexually ambiguous in phenotype

The observation that both male and female specific products are expressed in individual tumor cells indicates a sexually ambiguous identity. There are several mechanisms by which this might occur. One possibility is that a tumorous cell represents simultaneous male and female (intersexual) differentiation. This could occur if the ovarian tumor genes (e.g. *otu*, *Sxl* and *snf*) act to repress the activity of male-specific germ line genes, but are not essential for all female-specific gene expression. In this case, mutations that disrupt *otu*, *Sxl* or *snf* activity would allow both male and female germ line functions to be expressed, resulting in an intersexual phenotype (ie. ovarian tumors). This model is functionally analogous to the role played by the female-specific *doublesex* product in somatic sexual differentiation. The *doublesex* gene encodes for both male-specific and female-specific products that each act, at least in part, as negative regulators of gene expression (Baker and Ridge, 1980; Burtis and Baker, 1989). In *X/X* flies, the female *doublesex* product shuts down male-specific somatic gene expression allowing female differentiation to occur. In the absence of *doublesex* function, both male and female genes are expressed resulting in an intersexual phenotype (Baker and Ridge, 1980).

The proposal that tumors represent an intersexual phenotype is also consistent with observations suggesting that germ line sex determination requires both a germ line and a somatic contribution (Steinmann-Zwicky et al., 1989; Nöthiger et al., 1989; Oliver et al., 1993). Mutations in *otu*, *snf* and *Sxl* might eliminate the germ line component, thereby promoting male differentiation. However, because these cells are still developing in a female somatic background they might retain some female identity.

An alternative explanation for the expression of both male and female products in ovarian tumors is that the *otu*, *snf* and *Sxl* genes may have no role in the determination of sex in the germ line but are instead required for early stages in oogenesis. The *otu* gene, for example, has been implicated in the formation of the cytoplasmic connections between cystocytes (King, 1979; King and Storto, 1988). Similarly, *Sxl* may play a specific role in controlling cystoblast proliferation and in cystocyte differentiation (discussed below). By this model, the ovarian tumor cells still have a female identity but cannot progress into later stages of oogenesis. This would result in germ cells arrested at an early stage of oogenesis in the midst of unaffected somatic tissue that will continue to develop. This block in oogenesis may have unpredictable effects on germ line gene regulation, including the ectopic expression of some male-specific genes.

3.3. Neither Sxl, otu nor snf determine sexual identity in the germ line

Our data indicate that the *Sxl* gene plays a very different role in the germ line than soma. In somatic tissue, the X:A ratio determines whether the *Sxl* gene produces a functional product. All subsequent sexually dimorphic gene expression in the soma ultimately depends on the state of *Sxl* activity. In contrast, even though ovaries mutant for otu^{1}, $otu^{P\Delta 3}$ or snf^{1621} produce little to no germ line SXL protein (Bopp et al., 1993), both female-specific transcription and RNA processing still occur. Therefore the *Sxl* germ line function is not essential for female-specific germ line gene activity and, by this criteria, it cannot be the master gene for germ line sex determination. By similar reasoning, neither *otu* nor *snf* appear to control all aspects of germ line sex since mutations in either gene do not prevent female specific gene expression.

3.4. The role of otu and Sxl in regulating oogenesis

Our data indicate that the activity of the *otu* gene is not affected by either the loss or constitutive expression of *Sxl* (Figs. 2B and 3D). In contrast, the accumulation of a female germ line-specific *Sxl* RNA appears to depend, directly or indirectly, on the earlier expression of *otu* (Fig. 3A). These observations occurred coincident with experiments demonstrating that *otu* mutations can affect *Sxl* sex-specific splicing (Bopp et al., 1993; Oliver et al., 1993) and that a constitutive *Sxl* allele is epistatic to at least a subset of *otu* mutant phenotypes (Pauli et al., 1993; our data). Together these findings support, but do not prove, the hypothesis that the *otu* gene acts prior to *Sxl* in a pathway that regulates some aspects of oogenesis (Oliver et al., 1993; Pauli et al., 1993).

However, this model for *otu-Sxl* interactions may be overly simplistic. While it was shown that the constitutive expression of *Sxl* can suppress the mutant phenotype of some loss-of-function *otu* alleles (Pauli et al., 1993), only hypomorphic *otu* alleles are suppressable and in every case only partial suppression is obtained. Why is the suppression of *otu* by *Sxl* both allele dependent and incomplete? Furthermore, even the statement that *otu* acts upstream of *Sxl* is equivocal since both *otu* and *Sxl* produce multiple products and are required at early and late stages of oogenesis. Therefore, different *otu* functions temporally act before, after and perhaps even concurrent with *Sxl* activity.

In order to clarify the pattern of interaction between *Sxl* and *otu*, we did genetic epistasis experiments with *otu* alleles that affect specific OTU isoforms. From these data, we hypothesize a mechanism in which *Sxl* acts downstream of one OTU isoform and in parallel with the other OTU product (Fig. 6). Early stages in oogenesis require *otu* for the proliferation or viability of the female oogonial cells (King et al., 1986; Storto and King, 1987; Geyer et al., 1993). We propose that the 98-

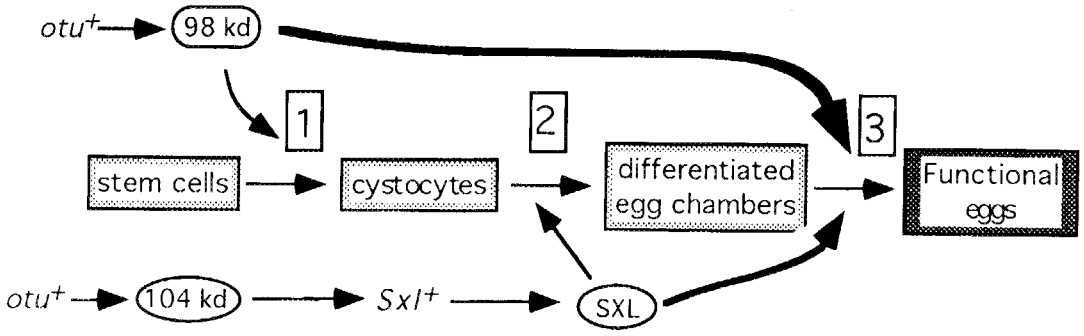


Fig. 6. Proposed roles of the *Sxl* germ line function and the *otu* isoforms in the regulation of oogenesis. This model proposes that the 98-kD OTU product acts early for the proliferation of cystocytes and later for the maturation of the egg cysts. The 104-kD product acts to regulate *Sxl* function. *Sxl* is required for the differentiation of cystocytes into the nurse cells and oocytes. *Sxl* is also needed for later stages in oogenesis. The absence of the 98-kD product will prevent stem cell and cystoblast proliferation, resulting in an agametic phenotype (1). Mutations in the 104-kD isoform or *Sxl* will result in a block at (2) which will give rise to ovarian tumors. Mutations that allow moderate levels of the 98-kD OTU isoform and *Sxl* activity will allow egg development past (1) and (2) but can now result in arrest at (3). This would give rise to the late stage oogenic arrest found with hypomorphic alleles of *otu* and *Sxl*. In these cases, the constitutive expression of *Sxl* can produce functional eggs only if there is sufficient 98-kD OTU activity.

kD OTU product is sufficient for this process in a pathway that is independent of *Sxl* activity. This is based on the observation that Sxl^{M1} does not suppress the mutant phenotype of *otu* alleles blocked in early stages of oogenesis (Pauli et al., 1993; our unpublished data). The 104-kD isoform is not needed for these early stages since otu^{13} mutants cannot produce detectable levels of the 104-kD polypeptide (Steinhauer and Kalfayan, 1992), but still show early oogonial proliferation and the development of tumorous egg chambers (King et al., 1986; Fig. 4).

The 104-kD OTU product is required for the activation of the *Sxl* germ line function (Fig. 6). Therefore, constitutive *Sxl* activity can suppress the ovarian tumor phenotype associated with mutations specific to the 104-kD isoform. Later stages of oogenesis involving the maturation of the egg chambers requires both *Sxl* activity and the 98-kD OTU isoform. This late requirement for *Sxl* is shown by the arrested late stage egg chambers obtained with *Sxl* mutations (Fig. 5B). That the 98-kD OTU isoform is also required at this stage is demonstrated by the finding that the degree of suppression by constitutive *Sxl* activity is influenced by the dosage of the 98-kD product (compare the effects of Sxl^{M1} on otu^{13}/otu^{2} and otu^{13}/otu^{10} with otu^{13}/otu^{13} in Table 2).

While the 104-kD OTU isoform appears to act upstream of *Sxl*, the same does not appear to be true for the 98-kD OTU product. otu^{7} and otu^{14} are hypomorphic mutations that are partially defective for both the 98-kD and 104-kD OTU products. otu^{2}/otu^{7} and otu^{2}/otu^{14} flies produce almost mature egg chambers, indicating that they must have more 104-kD OTU function than the predominantly tumorous otu^{13}/otu^{11} females. Even so, while the constitutive expression of *Sxl* can completely rescue the sterility of otu^{13}/otu^{11} females, it cannot suppress the mutant phenotype of otu^{2}/otu^{7} and otu^{2}/otu^{14} flies (Table 2). This supports the contention that the *Sxl* product cannot substitute for a defective 98-kD OTU isoform.

Our model is consistent with the temporal expression of the different *otu* isoforms during oogenesis (Steinhauer and Kalfayan, 1992). The 104-kD encoding RNA is expressed during the period when germ cells begin to differentiate into nurse cells and oocytes. This is the time we predict when female germ line differentiation would require the *Sxl* germ line product. The expression of the 98-kD product predominates later in oogenesis, during the period when the differentiated egg chambers are maturing, is consistent with it having a late function in oogenesis. Our model predicts the 98-kD product will also be expressed early in the development of the female germ line, perhaps during the embryonic and larval stages. This is currently under investigation.

4. Experimental procedures

4.1. Fly strains

The $otu^{P\Delta 3}$ allele is a deletion in the promoter region of the *otu* gene (Steinhauer and Kalfayan, 1992; Geyer et al., 1993). Sxl^{fs3} is a female-sterile hypomorphic allele of *Sxl* that gives rise to small tumorous ovaries (Oliver et al., 1990). Sxl^{f4} and Sxl^{f5} are female sterile alleles of *Sxl*. They were previously designated Sxl^{fs1} and Sxl^{fs2}, respectively. Descriptions of the other mutant loci are found in Table 2 and Lindsley and Zimm (1992).

4.2. Culture conditions and Feulgen staining

Fly cultures were kept under uncrowded conditions at 25°C. Female flies of the appropriate genotypes were aged 2–3 days after eclosion at 25°C. The ovaries were dissected in PBS, then stained by Feulgen reaction using a modification of the procedure described in Galigher and Kozloff (1971). Ovaries were hand dissected and fixed in Carnoy's solution (1:4 acetic acid: ethanol) for 2–3 min, incubated in 1 N HCl for 3–4 min and stained by Feulgen reagent. Staining was stopped by a 5 min incubation in dilute sulfuric acid. The ovaries were dehydrated by a series of washes in 20%, 50%, 70%, 90% and 100% ethanol. The treated ovaries were cleared in xylene and mounted in permount.

4.3. RNA preparation

Ovaries were dissected in cold PBS and transferred to 1 ml of 1:1 homogenization buffer: phenol (homogenization buffer: 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.0), 0.5% sodium dodecyl sulfate). The ovaries were immediately disrupted in a 2 ml Dounce homogenizer. The homogenate was extracted twice with 1:1 phenol: chloroform, then ethanol precipitated. The nucleic acid precipitate was resuspended in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and the concentration determined by ultraviolet absorbance spectrophotometry (O.D. 260).

4.4. RNA blotting and hybridization

Northern analyses were done as described in Geyer et al. (1993). Total ovarian RNA, 20 μg per lane, was loaded in an agarose/fomlaldehyde gel and blotted to Nytran (Schleicher and Schuell) filters by vacuum blotting (Hoefer). The RNA was cross-linked to the membranes by exposure to short-wave ultraviolet radiation in a UV multilinker (Ultra-Lum). Filters were prehybridized for 1 h at 42°C in 5× SSC, 4× Denhardt's solution, 50 μg/ml salmon sperm DNA, 0.2% SDS; then hybridized in 5× SSC, 4× Denhardt's solution, 50 μg/ml salmon sperm DNA, 0.2% SDS, 10% dextran sulfate and [^{32}P]-labeled probe at 42°C.

The probes were generated by random-priming using [^{32}P]-labeled dCTP. The probe for the *Sxl* RNAs was a 3.4-kb genomic fragment that detects two *Sxl* transcripts in wild-type ovaries; a 4.1-kb female-specific transcript (fem) present in the soma and probably the germ line, and a 3.1-kb female germ line-dependent RNA (gld) (Probe 'j' from Salz et al., 1989). This probe does not detect a number of other *Sxl* transcripts (Salz et al., 1989; Samuels et al., 1991). The *otu* transcript was identified by hybridization to a 2.0-kb cDNA probe specific for the *otu* transcript (Geyer et al., 1993). The *orb* probe was derived from a 2.1-kb *Eco*RI genomic fragment that hybridized to both the male and female-specific *orb* RNAs (Lantz et al., 1992). The *ovo*-specific probe was derived from a 0.7-kb *Xho*I fragment that contains sequences from exon 3 and exon 4 (Mevel-Ninio et al., 1991; Garfinkel et al., 1992). This probe fragment does not contain *ovo* sequences believed to be homologous to a repetitive sequence (Garfinkel et al., 1992). The Northern blots were hybridized to genomic *rp49* probe as a control for the amount of RNA loaded (O'Connell and Rosbash, 1988).

4.5. Construction of the constitutive Sxl, otu⁻ chromosomes

Sxl^{M1} was crossed on to chromosomes carrying otu^{2} and otu^{13}. The identification of the appropriate recombinant chromosomes was initially based on the presence of diagnostic flanking markers. The presence of Sxl^{M1} was confirmed both genetically, by its male lethal phenotype, and by Southern blot analysis (data not shown). Sxl^{M1} is associated with the insertion of a transposon near the sex-specific *Sxl* exon. This results in a characteristic 5.5-kb *Sal*I fragment (Maine et al., 1985). The presence of the *otu* allele was demonstrated by its inability to complement an *otu* null mutation.

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