

## Genetic and Molecular Characterization of *P* Element-Induced Mutations Reveals That the *Drosophila ovarian tumor* Gene Has Maternal Activity and a Variable Null Phenotype

Pamela K. Geyer,\* J. Scott Patton,\* Christopher Rodesch<sup>†</sup> and Rod N. Nagoshi<sup>†</sup>

\*Department of Biochemistry and <sup>†</sup>Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242

Manuscript received July 13, 1992

Accepted for publication October 8, 1992

### ABSTRACT

The mutations in the *ovarian tumor* (*otu*) gene arrest oogenesis at several stages in development. A series of deletion mutations in the *otu* region were characterized, each of which causes the absence or reduction of the *otu* transcript. These alleles range from the most severe class, which results in ovaries lacking egg cysts, to relatively mild mutations that allow the development of late stage oocytes. Heteroallelic combinations of these mutations demonstrate that the phenotypic complexity of *otu* mutant ovaries is due to a dosage dependent requirement for *otu* activity. Reciprocal cross and developmental Northern blot studies suggest a maternal requirement for *otu* in the development of the female germline. In addition we demonstrate that the *otu* zygotic null phenotype is variable, ranging from the absence of cysts in the most extreme cases, to the presence of tumorous egg chambers.

THE development of the female germline in *Drosophila* is a complex process requiring the activity of both somatic and germline-specific genes [reviewed in PAULI and MAHOWALD (1990)]. A number of mutations have been isolated that block egg development at different stages, resulting in female sterility (GANS, AUDIT and MASSON 1975; PERRIMON *et al.* 1986). One distinctive class of female sterile mutations is characterized by the "ovarian tumor" phenotype, in which mutant egg cysts undergo supernumerary cell divisions (PAULI and MAHOWALD 1990). Proposed causes for the production of ovarian tumors include defective cytokinesis (KING and STORTO 1988) and disruption of sex determination in the germline (PAULI and MAHOWALD 1990).

Extensive morphological studies provide a detailed description of oogenesis (KING 1970; MAHOWALD and KAMBYSELLIS 1980). The adult ovary consists of two lobes that are each composed of 15–20 ovarioles. At the tip of each ovariole are 2–3 mitotic stem cells that are descended from the embryonic pole cells. These cells divide into a daughter stem cell and a cystoblast. The cystoblast undergoes four mitotic divisions, characterized by incomplete cytokinesis, to produce 16 cystocytes connected by intercellular bridges. One of these cystocytes differentiates into an oocyte, the other 15 cystocytes become nurse cells. Somatic derived follicle cells surround the oocyte and nurse cells to form an egg cyst. Maturation of the egg cyst is subdivided into 14 stages that are distinguished by morphological criteria. Oogenesis occurs throughout adulthood and all 14 stages of development are present within each ovariole, arranged in a linear array.

Essential to oogenesis is the activity of the *ovarian tumor* (*otu*) gene. The *otu* product is required at multiple times during oogenesis, as demonstrated by the observation that *otu* mutations cause a range of morphological defects (KING and RILEY 1982). The *otu* mutations have been subdivided into three classes based on the average morphology of the mutant egg cysts in individual ovarioles (KING *et al.* 1986). Mutations of the quiescent (QUI) class are defective in oogonial proliferation or survival, resulting in ovarioles lacking cysts. Oncogenic (ONC) lesions produce ovarioles containing what are described as tumorous egg cysts. In this case, the mutant cystocytes undergo increased cell division such that each cyst contains up to several hundred cells (KING and RILEY 1982; BISHOP and KING 1984; KING and STORTO 1988). These divisions differ from normal cystoblast growth in that they are characterized by complete cytokinesis and the absence of intercellular bridges. The differentiated (DIF) class of mutations is characterized by ovarioles that contain more mature egg cysts, as defined by the presence of nurse-like cells and yolk deposition.

The isolation and the initial molecular characterization of the *otu* gene have been previously described (MULLIGAN, MOHLER and KALFAYAN 1988; CHAMPE and LAIRD 1989; STEINHAEUER, WALSH and KALFAYAN 1989). Germline transformation studies identified a 5.0-kb genomic DNA fragment that rescues the female sterility caused by *otu* mutations. This region produces at least one ovary-specific 3.2-kb RNA class as determined by Northern blot analysis (MULLIGAN, MOHLER and KALFAYAN 1988; COMER, SEARLES and

KALFAYAN 1992). Isolations of cDNAs indicate that at least two alternatively spliced RNAs of approximately the same size are produced from this gene (STEINHAEUER and KALFAYAN 1992; COMER, SEARLES and KALFAYAN 1992). These RNAs encode *otu* protein isoforms that differ in their temporal expression (STEINHAEUER and KALFAYAN 1992). It was proposed that these isoforms are required at different stages of oogenesis and may explain, in part, the phenotypic complexity of the *otu* mutations. Both the *otu* RNAs (PARKS and SPRADLING 1987) and proteins (STEINHAEUER and KALFAYAN 1992) are expressed in germ cells of the adult ovary. This tissue localization is consistent with the cell autonomous, germline requirement for *otu* activity (WIESCHAUS, AUDIT and MASSON 1981; PERRIMON and GANS 1983).

A model was developed to explain the phenotypic complexity of *otu* mutations. STORTO and KING (1987) proposed that *otu* activity is required at six developmental periods during oogenesis in a dosage dependent manner. During the first period, the *otu* product was hypothesized to be required at a relatively low concentration for the division of oogonia. Mutations that reduce the level of *otu* activity below this threshold result in the quiescent phenotype. Period 2 occurs when stem cells divide to form cystoblasts and sister stem cells. It was hypothesized that ONC alleles produce sufficient *otu* activity for stem cell division but not for cystoblast differentiation, leading to the production of tumorous egg cysts. The third period occurs during nurse cell differentiation. If the level of *otu* product falls below this third threshold, cells form that are morphologically reminiscent of nurse cells, but differ in that their chromosomes are polytene (KING *et al.* 1981). These mutant cells are denoted as pseudonurse cells (PNCs). Three additional periods were proposed to account for mutations that disrupt later stages of oogenesis, requiring either higher levels of the early *otu* product or the activity of a second *otu* function. Mutations that arrest oogenesis at the third or later thresholds result in the DIF class of mutant ovaries.

This model is based on extensive studies of over 100 heteroallelic combinations of 17 EMS-induced *otu* alleles (KING and RILEY 1982; KING *et al.* 1986; STORTO and KING 1987). One difficulty with these experiments is that molecularly uncharacterized *otu* mutations were used. Even the most severe *otu* alleles examined in these studies showed partial complementation in certain combinations, suggesting that they were not true null mutations (KING *et al.* 1986; KING and STORTO 1988). Therefore, the different phenotypes of the heteroallelic combinations examined could result by several different mechanisms, including a reduction in the levels of *otu* function or partial complementation between mutant or multiple *otu*

products. This ambiguity complicates the interpretations of these genetic experiments.

In this paper, we reexamine the mechanism of *otu* function using a set of deletions that are either localized to the *otu* promoter and noncoding regions or remove large portions of structural sequences as well. We have used these alleles in a series of *inter se* crosses to test hypotheses of multiple *otu* functions and threshold requirements during oogenesis. Our studies demonstrate that: (1) the full range of *otu* mutant cyst phenotypes can be obtained by reducing the level of *otu* product, supporting the proposal that *otu* activity is required in a dosage-dependent manner at different stages in oogenesis; (2) *otu* alleles display a reciprocal cross effect that is consistent with a maternal function in oogenesis; and (3) female germ cells can produce egg cysts even when deleted for the *otu* gene, suggesting that the zygotic *otu* function is redundant to either another gene or to the maternal *otu* contribution.

## MATERIALS AND METHODS

**Fly strains:** The *otuPΔ* alleles were obtained by remobilization of the *otu*-associated transposon in *otu<sup>P3</sup>* using a hybrid dysgenic set of crosses (D. MOHLER, personal communication). The six *PΔ* mutant chromosomes used in this study were: *otu<sup>PΔ1</sup> v f*; *y cv otu<sup>PΔ2</sup>*; *otu<sup>PΔ3</sup> v f*; *y cv otu<sup>PΔ4</sup>*; *y cv otu<sup>PΔ5</sup>*; *y cv otu<sup>PΔ6</sup>*. Each was balanced over either *FM7*, *FM3*, or *FM6*. *Sxl<sup>f52</sup>* is a female-sterile allele of *Sxl* (MOHLER and CARROLL 1984; PERRIMON *et al.* 1986), that is also designated as *Sxl<sup>f5</sup>* (LINDSLEY and ZIMM 1992). Descriptions of mutations and balancer chromosomes not described in the text are found in LINDSLEY and ZIMM (1992).

**Classification of mutations:** The mutant phenotypes associated with the *otu* mutations and the procedure for classifying mutant ovarioles has been previously described (KING and RILEY 1982; KING *et al.* 1986). Ovarioles are divided into eight groups (A–H) depending on whether they contain: (A) no germ cells; (B) tumorous egg cysts; (C) egg cysts containing both tumorous cells and pseudonurse cells; (D) tumorous egg cysts and pseudonurse cell cysts; (E) pseudonurse cell cysts; (F) pseudonurse cell cysts and oocyte cysts; (G) only oocytes; (H) tumorous cysts and oocyte cysts. The distribution of ovarioles within these groups for the different *otu* alleles are found in APPENDIX A. These eight classifications are consolidated into four groups [quiescent (QUI), tumorigenic (TUM), pseudonurse cell (PNC), oocyte] by the formula: QUI = frequency of A; TUM = frequency of B + C/2 + D/2 + H/2; PNC = frequency of C/2 + D/2 + E + F/2; oocyte = frequency of F/2 + G + H/2 (KING *et al.* 1986). Pseudonurse cells are defined as cells with large nuclei resembling normal nurse cells, but containing polytene chromosomes (KING and RILEY 1982).

**Determination of ovarian morphology:** Fly cultures were kept under uncrowded conditions at 25°. Female flies of the appropriate genotypes were aged 2–3 days after eclosion at 25°. Ovaries were prepared 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. After fixation, the ovaries were incubated in 1 N HCl for 3–4 min. This was followed by staining in Feulgen reagent until the nuclei were appropriately stained. 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 stained ovaries were cleared by xylene and mounted in permount.

**Reciprocal crosses:** Six independent sets of reciprocal crosses were done consisting of four separate experiments with the combination of *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ4</sup>, and one experiment each with *otu*<sup>PΔ5</sup> and *otu*<sup>PΔ4</sup>, and *otu*<sup>PΔ5</sup> and *otu*<sup>PΔ6</sup>. Each set of reciprocal crosses was established and cultured identically to minimize the effects of growth conditions on the mutant ovarian phenotype. The reciprocal crosses were:

Crosses A–D:

$$\frac{y\ cv\ otu^{P\Delta 4}}{FM7} \times \frac{y\ cv\ otu^{P\Delta 2}}{Y} \text{ and } \frac{y\ cv\ otu^{P\Delta 2}}{FM7} \times \frac{y\ cv\ otu^{P\Delta 4}}{Y}$$

Cross E:

$$\frac{y\ cv\ otu^{P\Delta 4}}{FM7} \times \frac{y\ cv\ otu^{P\Delta 5}}{Y} \text{ and } \frac{y\ cv\ otu^{P\Delta 5}}{FM7} \times \frac{y\ cv\ otu^{P\Delta 4}}{Y}$$

Cross F:

$$\frac{y\ cv\ otu^{P\Delta 4}}{FM7} \times \frac{y\ cv\ otu^{P\Delta 2}}{Y} \text{ and } \frac{y\ cv\ otu^{P\Delta 2}}{FM7} \times \frac{y\ cv\ otu^{P\Delta 4}}{Y}$$

Experiments A, D, E and F were grown at 25°; experiments B and C were grown at room temperature. For each set of reciprocal crosses, mutant female adults were aged 2–4 days posteclosion before dissection. To avoid experimenter bias, the mutant ovaries were examined using a single blind protocol in which the experimenter had no knowledge of the genotype or maternal history of the sample being examined during the analysis of the mutant phenotype. The numerical data obtained from these experiments are found in APPENDIX B.

**RNA preparation:** RNA was extracted from embryos using the sodium dodecyl sulfate (SDS)-phenol technique (SPRADLING and MAHOWALD 1979). Poly(A)<sup>+</sup> RNA was selected by chromatography on oligo(dT)-cellulose (AVIV and LEDER 1972).

Ovaries were dissected in ice-cold PBS (130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 3 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O) and transferred to 1 ml of 1:1 homogenization buffer:phenol (homogenization buffer: 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl [pH 8.0], 0.5% SDS). 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 UV absorbance spectrophotometry (OD<sub>260</sub>).

**RNA blotting and hybridization:** Northern analyses were done as described in NAGOSHI *et al.* (1988). Samples of 5–10 μg of total ovarian RNA were loaded per lane in an agarose/formaldehyde gel and blotted to Nytran (Schleicher and Schuell) filters by vacuum blotting (Hoefler). The RNA was cross-linked to the membranes by exposure to shortwave UV radiation in a UV multilinker (Ultra-Lum). Filters were prehybridized for 1 hr at 42° in 50% formamide, 5× SSC, 4× Denhardt's solution, 50 μg/ml salmon sperm DNA, 0.2% SDS; then hybridized in 50% formamide, 5× SSC, 4× Denhardt's solution, 50 μg/ml salmon sperm DNA, 0.2% SDS, 10% dextran sulfate and <sup>32</sup>P-labeled, random primed probe at 42°. The probe used for the hybridization was a cDNA clone derived from the 3.2-kb *otu* transcript generously provided by L. SEARLES (Department of Biology, University of North Carolina).

**Isolation of genomic DNA from *otu* mutants:** High molecular weight DNA was isolated from homozygous female flies by homogenization in 0.03 M Tris-HCl (pH 8.0),

0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA, 0.5% Triton X-100, followed by filtration through Nitex (no. 15) to eliminate cuticle debris. Nuclei were pelleted by centrifugation, resuspended in 0.01 M Tris HCl (pH 8.0), 0.35 M NaCl, 0.1 M EDTA and lysed with 1% *N*-lauryl sarcosine. DNA was purified by treatment with Proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. Genomic Southern analysis of each of the *otu* mutations was carried out using standard procedures (MANIATIS, FRITSCH and SAMBROOK 1982).

**Cloning of mutant sequences:** Sequences corresponding to *otu*<sup>PΔ2</sup>, *otu*<sup>PΔ3</sup> and *otu*<sup>PΔ5</sup> were cloned by polymerase chain reaction (PCR) amplification of genomic DNA. Primers for amplification of *otu*<sup>PΔ2</sup>, *otu*<sup>PΔ3</sup> were *otu* 9 [5'-GGCAATTTGAAAAGCTTCTGGTACA-3'] and *otu* 10 [5'-GAAAGCACCGAGAGAAATAGAATTC-3']. For *otu*<sup>PΔ5</sup>, the primers were *otu* 10 and *otu* 7 [5'-TCT-GCTCGGCGATCACA-3']. The PCR amplification reaction was carried out in 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2 mM MgCl<sub>2</sub>, 10 μM of each primer, 0.2 mM dNTPs and 2.5 units of Taq polymerase (Perkin Elmer-Cetus). Sequences were amplified using 30 cycles of 94° for 1 min, 55° for 2 min and 72° for 3 min, followed by a final extension reaction at 72° for 10 min. PCR products were subcloned into pUC18. Genomic DNA sequences corresponding to *otu*<sup>P3</sup>, *otu*<sup>PΔ4</sup> and *otu*<sup>PΔ6</sup> were isolated from genomic libraries prepared as described in GEYER, SPANA and CORCES (1986).

## RESULTS

**Female-sterile mutations derived from a *P* element-induced *otu* allele:** The *otu*<sup>P3</sup> allele is a *P* element-induced mutation that causes reduced female fertility when homozygous, and complete sterility when heterozygous with a deletion of the *otu* locus (MULLIGAN, MOHLER and KALFAYAN 1988; D. MOHLER, personal communication). The mutation is associated with the insertion of a *P* element near the transcription start site of *otu* (MULLIGAN, MOHLER and KALFAYAN 1988; our data, Figure 1A). Mobilization of this *P* element in a hybrid dysgenic cross resulted in the generation of a number of derivative female-sterile *otu* alleles (D. MOHLER, personal communication).

**Molecular characterization of *otu*<sup>P3</sup>:** Previous Southern blot analysis of the *otu*<sup>P3</sup> mutation indicated that this allele is associated with an insertion of approximately 0.89 kb in the 1.0-kb *Eco*RI fragment located at the 5' end of the gene (MULLIGAN, MOHLER and KALFAYAN 1988). Isolation and characterization of mutant DNA from these flies identified the insertion as a 683-bp *P* element integrated near the mRNA start site in the same transcriptional orientation as *otu* (Figure 1).

To determine whether the *P* element insertion altered the normal start site of transcription, primer extension analysis was undertaken. Primer extension analysis of *otu*<sup>P3</sup> RNA generated several extension products that correspond to major start sites seen in wild-type flies (COMER, SEARLES and KALFAYAN 1992), with one additional site at +11 (numbering as in

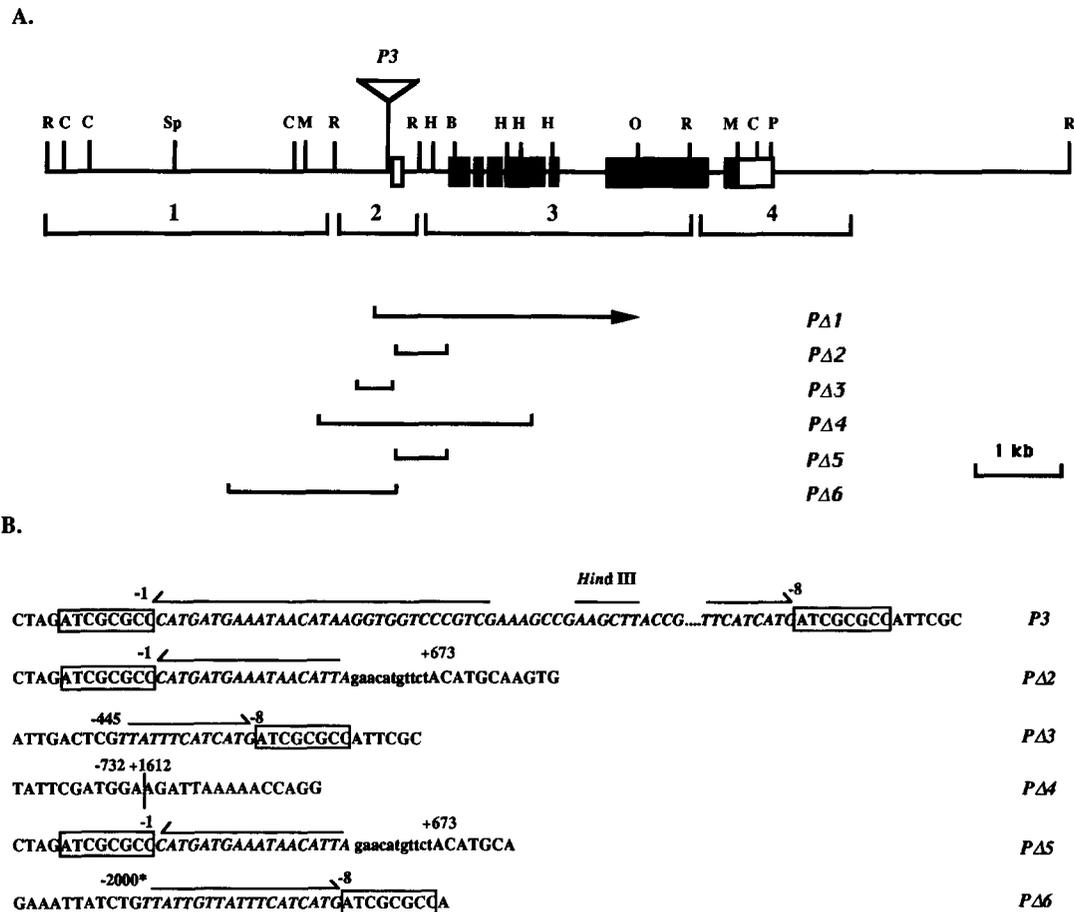


FIGURE 1.—Molecular characterization of *otu*<sup>P3</sup> and *otu*<sup>PΔ</sup> mutations. (A) The restriction map of the *otu* gene. Exons are indicated as boxes; solid boxes are the protein coding region. The position of the *P* element insertion in *otu*<sup>P3</sup> is indicated by the raised triangle. The positions of the *EcoRI* restriction fragments (probes 1–4) used to characterize the *otu*<sup>PΔ</sup> mutations are indicated directly beneath the restriction map (see Figure 2). Excision of the *P* element from *otu*<sup>P3</sup> caused deletion of sequences in each of the *otu*<sup>PΔ</sup> mutations. The extent of each deletion is presented. Restriction enzymes symbols are as follows: B, *Bam*HI; C, *Cla*I; R, *Eco*RI; H, *Hind*III; M, *Mlu*I; O, *Xho*I; P, *Pst*I; Sp, *Sph*I. (B) DNA sequences surrounding the *P* element insertion in *otu*<sup>P3</sup> or the breakpoints of the deletion in each *otu*<sup>PΔ</sup> mutation are listed. The *P* element in *otu*<sup>P3</sup> is integrated 9 bp upstream of a major transcription start site in the same transcriptional orientation as the *otu* gene. The deletions in *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ3</sup> are identical and have 673 bp removed, *otu*<sup>PΔ3</sup> is deleted for 455 nucleotides, approximately 2.3 kb are missing in *otu*<sup>PΔ4</sup>, and in *otu*<sup>PΔ6</sup> approximately 2 kb of sequences are removed. *otu* sequences are shown in upper case letters, *P* element sequences in upper case italics, and sequences of unknown origin are shown in lower case. Arrowheads indicate the position of the *P* element 31-bp inverted repeats. Boxed areas show sequences duplicated upon *P* element insertion.

COMER, SEARLES and KALFAYAN 1992; data not shown). The novel start site does not alter the coding region of the *otu* transcript.

**Molecular analysis of female-sterile alleles derived from *otu*<sup>P3</sup>:** Genomic Southern analysis of female-sterile alleles derived from *otu*<sup>P3</sup> indicated that each resulted from a deletion of *otu* sequences (Figure 2; data not shown). The largest deletion was associated with the *otu*<sup>PΔ1</sup> allele. The limits of this deletion were examined by hybridization of genomic DNA with several different regions of the *otu* gene (Figure 2). Hybridization with a probe containing the 5' end of the gene identifies a novel 9.4-kb *Eco*RI fragment in homozygous *otu*<sup>PΔ1</sup> flies (probe 2, Figure 2). Probes derived from sequences containing the coding portion of the *otu* gene do not hybridize to any *otu*<sup>PΔ1</sup> fragment (probes 3 and 4, Figure 2). From these results, we conclude that the *otu*<sup>PΔ1</sup> lesion is at least a 6.5-kb

deletion of the *otu* locus that completely removes *otu* coding sequences. Furthermore, these data demonstrate that *otu* coding sequences are not duplicated elsewhere in the *otu*<sup>PΔ1</sup> genome.

Genomic Southern analysis of *otu*<sup>PΔ4</sup> and *otu*<sup>PΔ6</sup> indicated that both carry deletions of approximately the same size, although each removes different portions of the *otu* gene (Figure 1A; data not shown). DNA corresponding to each mutation was cloned and sequenced. In *otu*<sup>PΔ4</sup> flies, a 2.3-kb sequence is excised, extending from -732 bp to +1612 from the start site of transcription (numbering as in COMER, SEARLES and KALFAYAN 1992). This deletion removes 5' regulatory sequences, the first four exons and most of the fifth exon (Figure 1A). The *otu*<sup>PΔ6</sup> deletion originates at one end of the *P* element and removes only 5' regulatory sequences, extending approximately 2 kb upstream of the start site of transcription (Figure 1A).

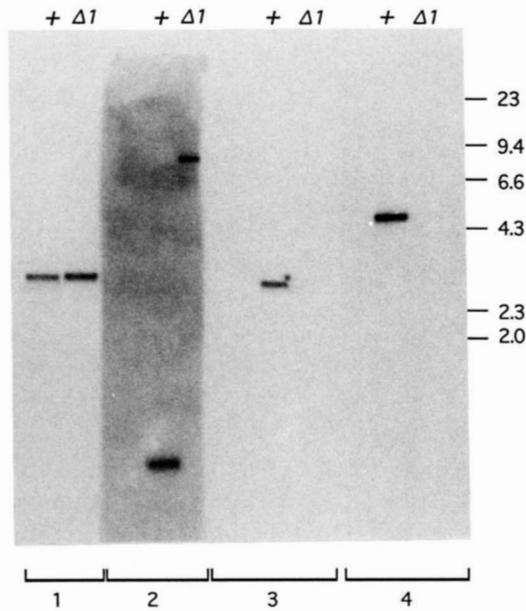


FIGURE 2.—Southern analysis of genomic DNA isolated from *otu*<sup>PΔ1</sup>. A sample of 5 μg of total genomic DNA isolated from either Oregon R (+) or *otu*<sup>PΔ1</sup> (Δ1) was digested with *Eco*RI and electrophoresed on a 1% agarose gel. Four Southern blots are shown which were hybridized with either probes 1, 2, 3 or 4. Location of the sequences used for probes are shown in Figure 1. The numbers at the side indicate the position of migration of the DNA size standards (λ DNA digested with *Hind*III).

Genomic Southern analysis of *otu*<sup>PΔ3</sup>, *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ5</sup> indicated that these mutations are also associated with deletions within the *otu* gene [STEINHAEUER and KALFAYAN (1992) and our data]. The *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ5</sup> mutations carry deletions of *otu* sequences originating within the 1.0-kb *Eco*RI fragment and terminating in the 3.2-kb *Eco*RI fragment. In *otu*<sup>PΔ3</sup>, only sequences within the 1.0-kb *Eco*RI fragment are removed (data not shown).

To more accurately map these mutations, each lesion was cloned and sequenced (Figure 1B). The *otu*<sup>PΔ3</sup> mutation removes 437 bp of 5' flanking DNA beginning at one end of the *P* element insertion, leaving intact the major start sites of transcription for the *otu* gene. DNA sequence analyses of *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ5</sup> demonstrate that these alleles are associated with an identical structural lesion. In both cases, *otu* sequences originating at the site of the *P* element insertion and extending to +673 from the start site of transcription are excised. This deletion removes the major wild-type transcriptional start sites and extends to the second exon just downstream of the wild-type translation start site. However, since a second in-frame translation start codon is found one codon later, we predict that the *otu* protein made in *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ5</sup> flies differs from wild-type only in removing two amino acids, the terminal methionine and histidine.

Although the molecular data suggest that *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ5</sup> alleles have identical deletions, they have been kept in separate stocks for a number of years and so may differ either in other portions of the *otu* locus or

in their genetic backgrounds. This could cause variations in their mutant phenotype and in their interactions with other alleles. For these reasons, we tested *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ5</sup> independently.

**PΔ mutations cause the reduction or absence of *otu* RNA accumulation:** Representative Northern blots are shown to demonstrate how the different *otu* structural rearrangements affect *otu* transcription (Figure 3). Total RNA preparations from wild-type and mutant ovaries were examined by Northern analyses. In these studies, only a single 3.2-kb, ovary-specific transcript was detected from the *otu* locus. We were not able to detect *otu* transcripts from female somatic tissue, whole males, or isolated testes (data not shown). Our data on the wild-type pattern of *otu* transcript accumulation are in agreement with the Northern analyses described by COMER, SEARLES and KALFAYAN (1992). Both studies, however, differ from the findings of MULLIGAN, MOHLER and KALFAYAN (1988). In addition to the 3.2-kb RNA, MULLIGAN, MOHLER and KALFAYAN (1988) reported additional RNAs from the *otu* region, including somatic and male-specific transcripts and multiple ovarian RNAs. The reasons for the discrepancies between these studies are unclear. Currently, no effect of *otu* mutations on somatic tissues or in males has been demonstrated, therefore the roles of the putative somatic and male-specific transcripts are unclear.

Ovaries from *otu*<sup>P3</sup> flies accumulate less *otu* transcript than wild-type, consistent with the reduced fertility associated with the *otu*<sup>P3</sup> allele (Figure 3A). No detectable transcripts were found for *otu*<sup>PΔ1</sup>, *otu*<sup>PΔ3</sup>, *otu*<sup>PΔ4</sup> or *otu*<sup>PΔ6</sup>, even after prolonged exposure (Figure 3, B–D). STEINHAEUER and KALFAYAN (1992) reported that *otu*<sup>PΔ3</sup> produced low but detectable amounts of one *otu* protein isoform, as determined by Western blot analysis. Either the *otu*<sup>PΔ3</sup> allele produces the *otu* transcript prior to the adult stage, or it is expressed at levels below our sensitivity of detection. For *otu*<sup>PΔ2</sup> and *otu*<sup>PΔ5</sup> a transcript that comigrates with the wild-type *otu* RNA is observed, but it is found at lower levels than in either wild-type or *otu*<sup>P3</sup> ovarian RNA (Figure 3E). This is consistent with studies demonstrating a reduced level of *otu* protein produced by the *otu*<sup>PΔ2</sup> allele (STEINHAEUER and KALFAYAN 1992).

To control for differences in loading and for the possibility that the mutations might have a general effect on transcription, the *otu* levels for each genotype were compared with the levels of the constitutively expressed ribosomal RNA *rp49* (O'CONNELL and ROSBASH 1984). In these and previous studies, we compared the total RNA loaded per lane (as determined by ethidium bromide staining and spectrophotometric measurements) with the amount of *rp49* transcript detected by hybridization (data not shown). The proportion of *rp49* RNA to total RNA was unaffected by the mutations used in this study. We

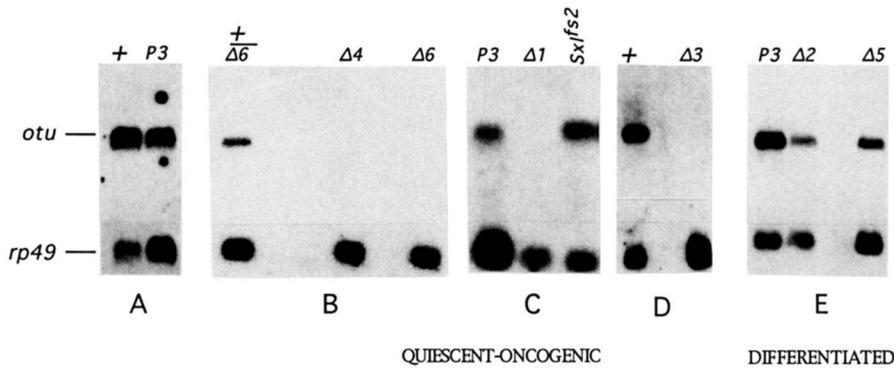


FIGURE 3.—Northern analysis of *otu* RNA from different *otu* mutant ovaries. A–E each represent separate Northern blots from different hybridizations. Samples of 10–20  $\mu$ g of total RNA from dissected ovaries were added to each lane. The ribosomal protein RNA, *rp49*, is used as a loading standard to compare the relative amounts of RNA loaded in each lane for each Northern blot. It should not be used to compare loadings between blots. For B, C and D longer exposures failed to detect *otu* transcripts from *otu*<sup>P $\Delta$ 4</sup>, *otu*<sup>P $\Delta$ 6</sup>, *otu*<sup>P $\Delta$ 1</sup> and *otu*<sup>P $\Delta$ 3</sup> ovaries. Symbols: (+) wild-type ovaries (two copies of *otu*<sup>+</sup>); (P3) *otu*<sup>P3</sup> ovaries; ( $\Delta$ 1– $\Delta$ 6) *otu*P $\Delta$  mutant ovaries.

therefore believe that *rp49* RNA levels accurately reflect the level of total RNA present in wild-type and *otu* mutant ovaries.

In addition, it is possible that differences in levels of *otu* transcript present in the various mutants result from a reduction in the amount and type of ovarian tissue present because *otu* mutants have smaller than normal ovaries and a decreased number of late stage egg cysts. Thus, these ovaries have a higher proportion of somatic cells to germ cells than do *otu*<sup>+</sup> ovaries. To control for this possibility, the accumulation of *otu* transcript was examined in flies homozygous for the mutation *Sxlf52* (MOHLER and CARROLL 1984; PERRIMON *et al.* 1986) (Figure 3C). This mutation has a phenotype similar to the ONC class of *otu* lesions, causing a reduction in ovarian tissue and the formation of tumorous egg cysts (see Figure 5I). We find that the *Sxlf52* mutation does not have a detectable effect on the levels of *otu* RNA relative to wild-type ovaries. In fact, the amount of *otu* RNA in *Sxlf52* mutant ovaries is greater than in *otu*<sup>P3</sup> ovaries (Figure 3C), even though the latter ovaries are larger and can produce functional eggs. Therefore, the absence or reduction in the level of *otu* transcript in the *otu*P $\Delta$  deletions is not attributable to a secondary effect of the abnormal ovary morphology, but is rather a direct result of the *otu*P $\Delta$  lesions.

**The *otu*<sup>P $\Delta$ 5</sup> deletion alters the start site of transcription relative to its parental strain and a wild-type Canton S strain:** Northern analyses of *otu*<sup>P $\Delta$ 5</sup> (and the identical *otu*<sup>P $\Delta$ 2</sup> lesion) indicate that this allele produces a wild-type sized RNA even though it is deleted for the major mRNA start sites. To determine the location of the start sites used by this deletion, primer extension analysis was done on *otu*<sup>P $\Delta$ 5</sup> ovarian RNA. Two extension products were obtained corresponding to start sites at –26 and –16 from the wild-type mRNA CAP site (data not shown). These start sites differ from those of the parental strain *otu*<sup>P3</sup> and correspond to minor start sites detected in wild type (data not shown).

**Deletions in the 5' portion of the *otu* gene can produce all classes of *otu* mutant phenotypes:** We

characterized the *otu*P $\Delta$  deletions according to their morphological phenotype in homozygous mutant ovaries (Figures 4 and 5). All six mutations cause female sterility when homozygous and have no effect in males. The morphologies of the ovary and germ cells were classified using the methodology of KING *et al.* (1986) (described in MATERIALS AND METHODS). The partially fertile parental strain, *otu*<sup>P3</sup>, has an almost wild-type morphology (Figure 5A), which contrasts with the distinctly mutant ovaries produced by the female-sterile derivatives of this allele (Figures 4 and 5, B–G). A correspondence exists between the size of the deletion and the severity of the phenotype. Deletions of large portions of the promoter and presumptive 5' regulatory regions (*otu*<sup>P $\Delta$ 4</sup>, *otu*<sup>P $\Delta$ 1</sup>, *otu*<sup>P $\Delta$ 6</sup>) gave the most severe phenotype, resulting in a substantial proportion of ovarioles that lacked egg cysts (Figures 4 and 5, B and C). In contrast, *otu*<sup>P $\Delta$ 3</sup>, a smaller deletion partially overlapping *otu*<sup>P $\Delta$ 4</sup>, resulted in few quiescent ovarioles and a predominance of the tumorigenic class with the occasional formation of more mature cysts (Figures 4 and 5F). The least severe alleles *otu*<sup>P $\Delta$ 2</sup> and *otu*<sup>P $\Delta$ 5</sup> result in large ovaries containing primarily late stage egg cysts characterized by pseudonurse cells and frequently yolky oocytes (Figures 4 and 5, G and H). Molecular mapping and Northern analysis predict that these mutants will produce a near wild-type protein but at a reduced level. These data suggest that a DIF *otu* mutant phenotype may arise from a reduction in the level of *otu* product, as suggested by KING and STORTO (1988), and does not depend on a specific inactivation of a late *otu* function.

**The null phenotype of *otu* is variable:** In previous studies, it was found that heteroallelic combinations between the most severe *otu* alleles resulted in a mutant ovary phenotype that was less severe than either homozygote (KING *et al.* 1986). This indicates that even these severe alleles produced some product that could interact beneficially in the heterozygote (KING and STORTO 1988), hence these alleles may not be true null mutations. To unambiguously determine the null phenotype of *otu*, we examined *otu*<sup>P $\Delta$ 1</sup> homozy-

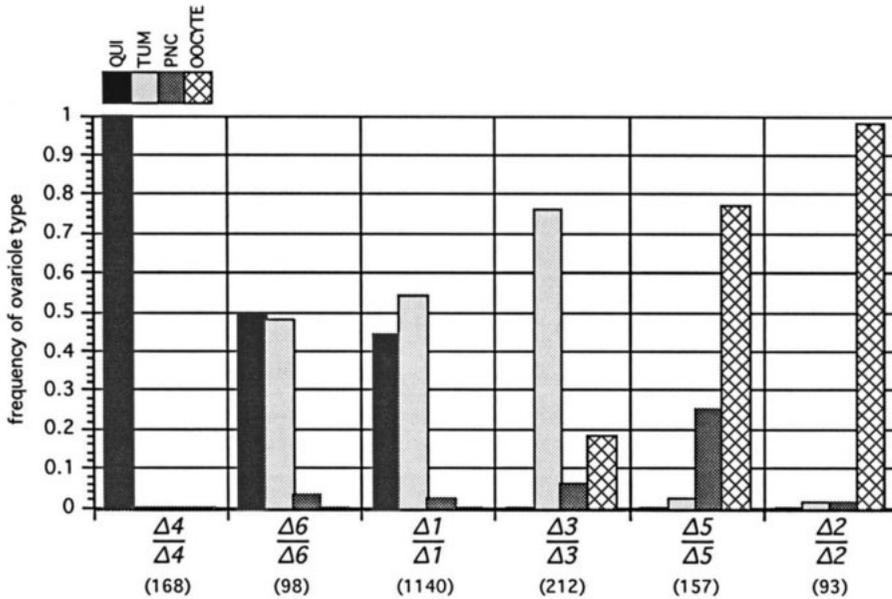


FIGURE 4.—Mutant ovarian phenotypes of the *otuPΔ* alleles. The six *otuPΔ* alleles were examined as homozygotes for their mutant ovarian phenotype. Mutant ovarioles were characterized as described by KING *et al.* (1986). Symbols: QUI, ovarioles lacking egg cysts; TUM, ovarioles containing tumorous egg cysts; PNC, ovarioles containing egg cysts with pseudonurse cells and no oocyte; OOCYTE, ovarioles containing mature egg cysts. The numbers in parentheses indicate the total number of ovarioles examined. The numerical data used in this figure are found in APPENDIX A.

gotes. The *otu<sup>PΔ1</sup>* mutation deletes the entire *otu* coding region and therefore must represent a functionally null allele (Figure 1A). Homozygous mutant females contain tumorous egg cysts and, in general, have a less severe average phenotype than *otu<sup>PΔ4</sup>* homozygotes (Figures 4 and 5D). When *otu<sup>PΔ1</sup>* was made heterozygous with a deletion of the entire *otu* locus, *Dff1JRA2*, similar tumorous egg cysts were obtained (Figure 5K). Surprisingly, even more mature cysts containing pseudonurse cells can develop in *otu<sup>PΔ1</sup>* homozygotes (Figure 5E). These egg cysts are rare and generally contain fewer pseudonurse cells than found in the more mild *otu* alleles.

A similar result is obtained with the *otu<sup>17</sup>* mutation. This allele is associated with a large deletion of part of the *otu* coding region (MULLIGAN, MOHLER and KALFAYAN 1988; data not shown) and so is likely to be a null mutation. Ovaries mutant for this allele may also contain tumorous egg cysts (Figure 5J), again indicating that even in the absence of *otu* product in the zygote, germline cells survive and proliferate to produce abnormal egg cysts.

**Different *otu* mutant classes may result from changes in the level of *otu* product:** We tested the dosage model of STORTO and KING (1987) by examining heteroallelic combinations of the *otuPΔ* mutations that we predict contain reduced levels of *otu* product. The *otu<sup>PΔ2</sup>* and *otu<sup>PΔ5</sup>* alleles are the least severe of the *otuPΔ* lesions, both of which produce DIF class ovaries when homozygous. Each of these alleles was made heterozygous with a more severe *PΔ* allele and the ovarian phenotype of the different combinations was examined (Figure 6). A dramatic shift is observed in the relative proportions of oocyte and pseudonurse cell ovarioles. In the *otu<sup>PΔ2</sup>* and *otu<sup>PΔ5</sup>* homozygous ovaries, at least 75% of the ovarioles contain mature oocyte egg cysts in which de-

tectable yolk deposition had occurred. In contrast, when either *otu<sup>PΔ2</sup>* or *otu<sup>PΔ5</sup>* is heterozygous with an oncogenic *PΔ* allele (*otu<sup>PΔ1</sup>* or *otu<sup>PΔ3</sup>*), the majority of ovarioles contain PNC cysts. Even more severely affected ovarioles are detected in combinations with the quiescent allele *otu<sup>PΔ4</sup>*.

Particularly relevant to the dosage model are the heteroallelic combinations of the DIF alleles with the functionally null deletions, *otu<sup>PΔ1</sup>* and *otu<sup>PΔ4</sup>*. Our molecular data indicate that neither allele can produce a mutant *otu* product. Therefore, the more severe ovarian phenotype that occurs when *otu<sup>PΔ2</sup>* or *otu<sup>PΔ5</sup>* are made heterozygous with *otu<sup>PΔ1</sup>* (or *otu<sup>PΔ4</sup>*) (Figure 6), must result from the reduction in the level of *otu* product from *otu<sup>PΔ2</sup>* or *otu<sup>PΔ5</sup>*. These data are consistent with the dosage model, indicating that differences between tumorous egg cysts and more mature oogenic stages can be attributed to changes in the levels of *otu* activity.

In each of the crosses described in Figure 6, the mutant daughters were derived from mothers carrying the less severe *otu* allele (*otu<sup>PΔ2</sup>* or *otu<sup>PΔ5</sup>*). This was done to control for the reciprocal cross effects that are described in the next section.

**Reciprocal cross differences suggest a maternal function for *otu*:** Reciprocal crosses experiments were done in which the two most severe *otuPΔ* alleles (*otu<sup>PΔ4</sup>* and *otu<sup>PΔ6</sup>*) were mated to the two least severe alleles (*otu<sup>PΔ2</sup>* and *otu<sup>PΔ5</sup>*). In the first direction, females were heterozygous for a severe allele of *otu* (*otu<sup>PΔ4</sup>* or *otu<sup>PΔ6</sup>*) and an *otu<sup>+</sup>* balancer chromosome. These were mated to males carrying a mild *otu* mutation (*otu<sup>PΔ2</sup>* or *otu<sup>PΔ5</sup>*). The ovaries of the *otu<sup>-</sup>* daughters were then characterized by the numbers of cysts containing (i) tumorous cells, (ii) pseudonurse cells, (iii) oocytes and yolk deposition (Figure 7). These results were compared to ovaries of daughters of the identical geno-

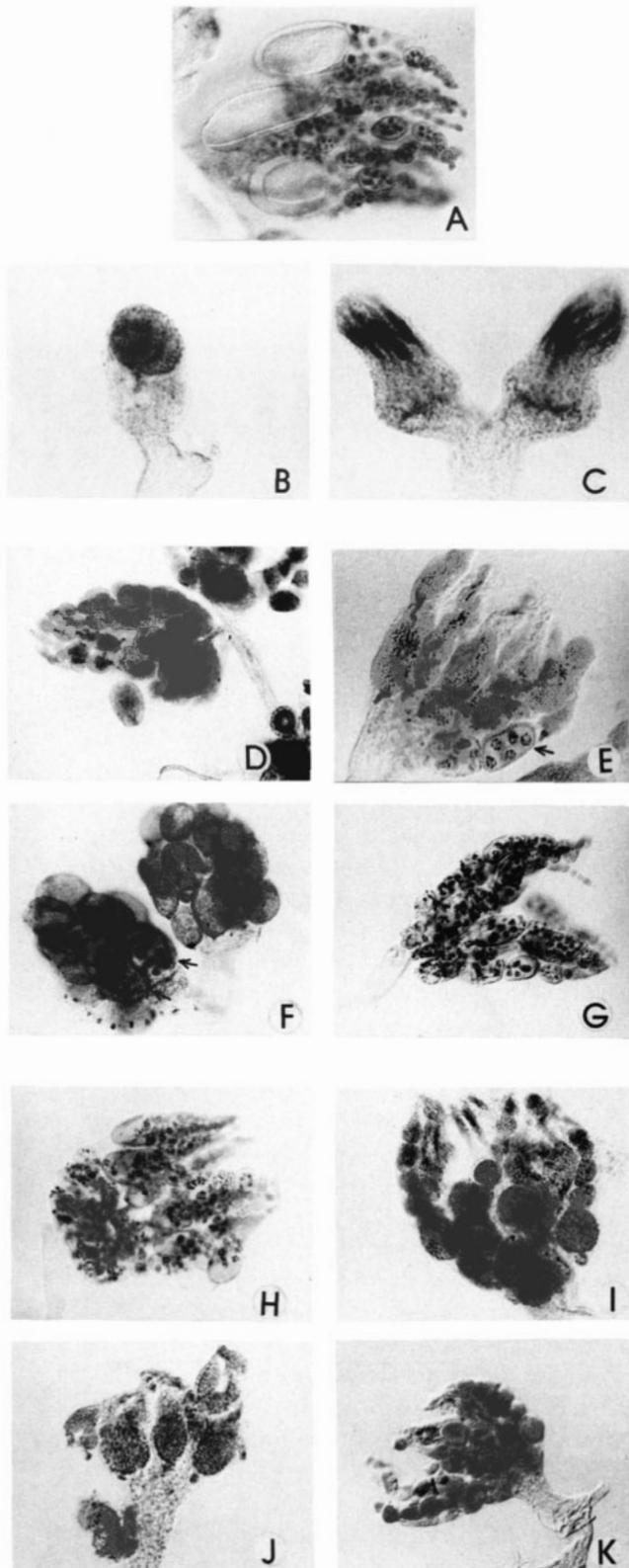


FIGURE 5.—Photomicrographs of Feulgen-stained mutant ovaries. Mutant phenotypes representative of each *otuPΔ* allele are shown. Flies were grown at room temperature and aged for 2–3 days before dissection. Ovarian cell nuclei are stained by Feulgen reaction. (A) ovary from *otu<sup>P3</sup>* homozygotes. These flies are fertile and display a near wildtype phenotype. (B) *otu<sup>PA6</sup>* mutant ovarioles are either quiescent or tumorous. The ovaries contain few egg cysts. (C) *otu<sup>PA4</sup>* mutant ovaries are the most severe of the *otuPΔ* mutations

type, in which the mild allele (*otu<sup>PA2</sup>* or *otu<sup>PA5</sup>*) was maternally contributed.

Our data demonstrate that when the more extreme *otu* allele is contributed by the mother, a more severe *otu* phenotype is found in the mutant daughters (Figure 7). The strongest effect is most consistently seen with crosses between *otu<sup>PA4</sup>* and *otu<sup>PA2</sup>*. Four independent experiments were done with this set of alleles (crosses A–D, Figure 7). In each case the frequency of tumorous cysts increases and the frequency of mature oocytes decreases when the mother carries the more severe *otu* allele. A similar pattern was observed for the other allelic combinations tested (crosses E and F, Figure 7).

There is substantial variability in the reciprocal differences found in each of these experiments, even in those cases where the same alleles are being tested (compare crosses A–D, Figure 7). We believe that this is due to our inability to completely eliminate the maternal *otu* contribution in these crosses. Because *otu* activity is required in the female germline for functional eggs, all mothers had to carry at least one copy of *otu<sup>+</sup>*. Thus, only relatively small changes in the maternal contribution of *otu* are examined, which might easily be influenced by differences in genetic background and environmental effects. Despite this difficulty, a consistent pattern of reciprocal cross differences is obtained for each of the allelic combinations tested, indicating that small changes in the maternal contribution of *otu<sup>+</sup>* can have detectable effects on oogenesis.

While mutations in *otu* affect both egg cyst differentiation and numbers, we observe reciprocal cross differences only in the morphology of the egg cysts. In the same set of crosses as described in Figure 7, the number of egg cysts was determined for each mutant ovary (Figure 8). While there is variation in ovary size between reciprocal pairings, it does not correlate with the maternal contribution. These data suggest that the role of *otu* in the viability and proliferation of germ cells is separable from its requirement in egg cyst maturation, the former being less dependent on a maternal contribution of *otu*.

Further evidence for a maternal function for *otu* was obtained by examining *otu* expression during embryonic development. Results of Northern blot

with few if any germ cells. (D and E) *otu<sup>PA1</sup>* mutant ovaries contain predominantly quiescent and tumorous ovarioles. The phenotype is sensitive to growth conditions. Rarely, more mature egg cysts containing pseudonurse cells can develop (E, arrow). (F) *otu<sup>PA3</sup>* mutant ovaries contain predominantly tumorous cysts but occasionally more mature cysts are seen (arrows). (G) *otu<sup>PA2</sup>* and (H) *otu<sup>PA5</sup>* are mild *otu* alleles resulting in ovaries containing late stage oocytes. (I) *Sxl<sup>f/2</sup>* mutant ovaries are generally small, with predominantly tumorous egg cysts. (J) *otu<sup>I7</sup>* causes mostly quiescent or tumorous ovarioles, resembling the *otu<sup>PA6</sup>* and *otu<sup>PA1</sup>* mutant phenotypes. (K) *otu<sup>PA1</sup>/Df(1)RA2* ovaries are hemizygous for *otu<sup>PA1</sup>*. The mutant phenotype is similar to *otu<sup>PA1</sup>* homozygotes.

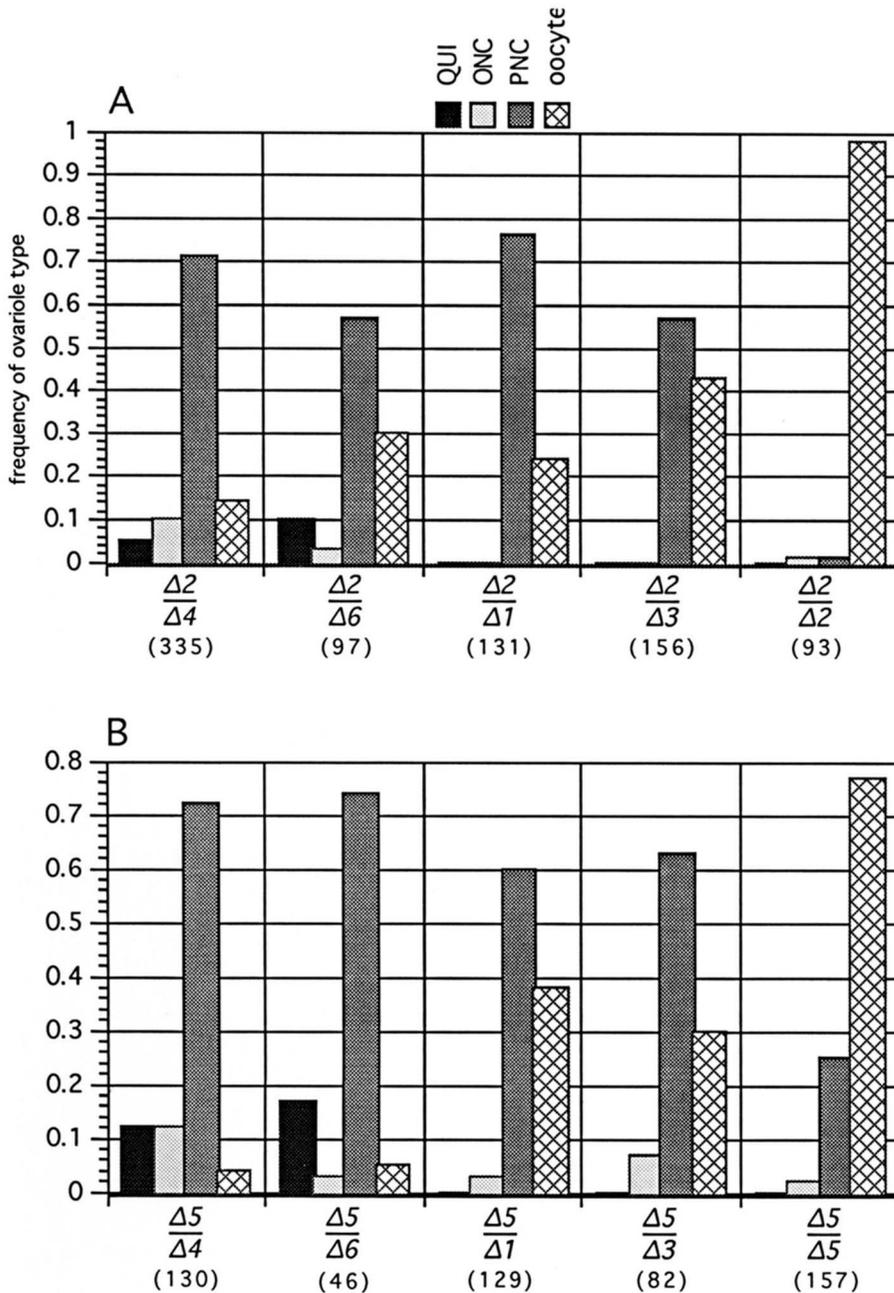


FIGURE 6.—Mutant ovarian phenotypes from different *otu* allelic combinations. The two DIF *otuPΔ* alleles were examined in combination with each of the more severe *otu PΔ* mutations. In (A), *y cv otu<sup>PΔ2</sup>/otuPΔ* daughters from the cross, *y cv otu<sup>PΔ2</sup>/FM7 × otuPΔ/Y*, were examined for their ovarian phenotype. The same design was used in (B) to test *y cv otu<sup>PΔ5</sup>*. In each experiment the DIF allele was maternally contributed. Mutant ovarioles were characterized as described by KING *et al.* (1986). Numbers in parentheses indicate the number of ovarioles examined. Symbols: QUI, ovarioles lacking egg cysts; ONC, ovarioles containing tumorous egg cysts; PNC, ovarioles containing egg cysts with pseudonurse cells and no oocyte; oocyte, ovarioles containing mature egg cysts. The numerical data used in this figure are found in APPENDIX A.

analysis of *otu* RNA isolated from staged embryos detected *otu* mRNA in 0–4-hr embryos which completely disappears in 4–6-hr embryos (Figure 9).

DISCUSSION

**Different levels of *otu* function are required during oogenesis:** The *ovarian tumor* gene plays a crucial role in *Drosophila* oogenesis, with an apparent requirement at multiple stages in germ cell development. Mutant phenotypes associated with *otu* lesions range from an absence of egg cysts, to tumorous egg cysts, to arrested late stage oogenesis. Does this phenotypic complexity result from multiple *otu* products, each with distinct functions, or is a single *otu* function required at different stages in development at different levels of activity? Previous genetic studies showed

that heteroallelic combinations of severe and mild *otu* mutations generally result in an intermediate phenotype, lending support for the model that *otu* activity is required in a dosage dependent manner (KING *et al.* 1986; STORTO and KING 1987). However, because these experiments were done with molecularly uncharacterized alleles, alternative interpretations exist for these results. These include the possibility that the intermediate *otu* phenotypes result from differential inactivation of two or more *otu* products, or that different mutations produced altered polypeptides that interact to give novel function. The former possibility is particularly relevant in view of recent studies demonstrating the existence of two *otu* protein isoforms that result from alternative RNA splicing (STEINHAEUER and KALFAYAN 1992).

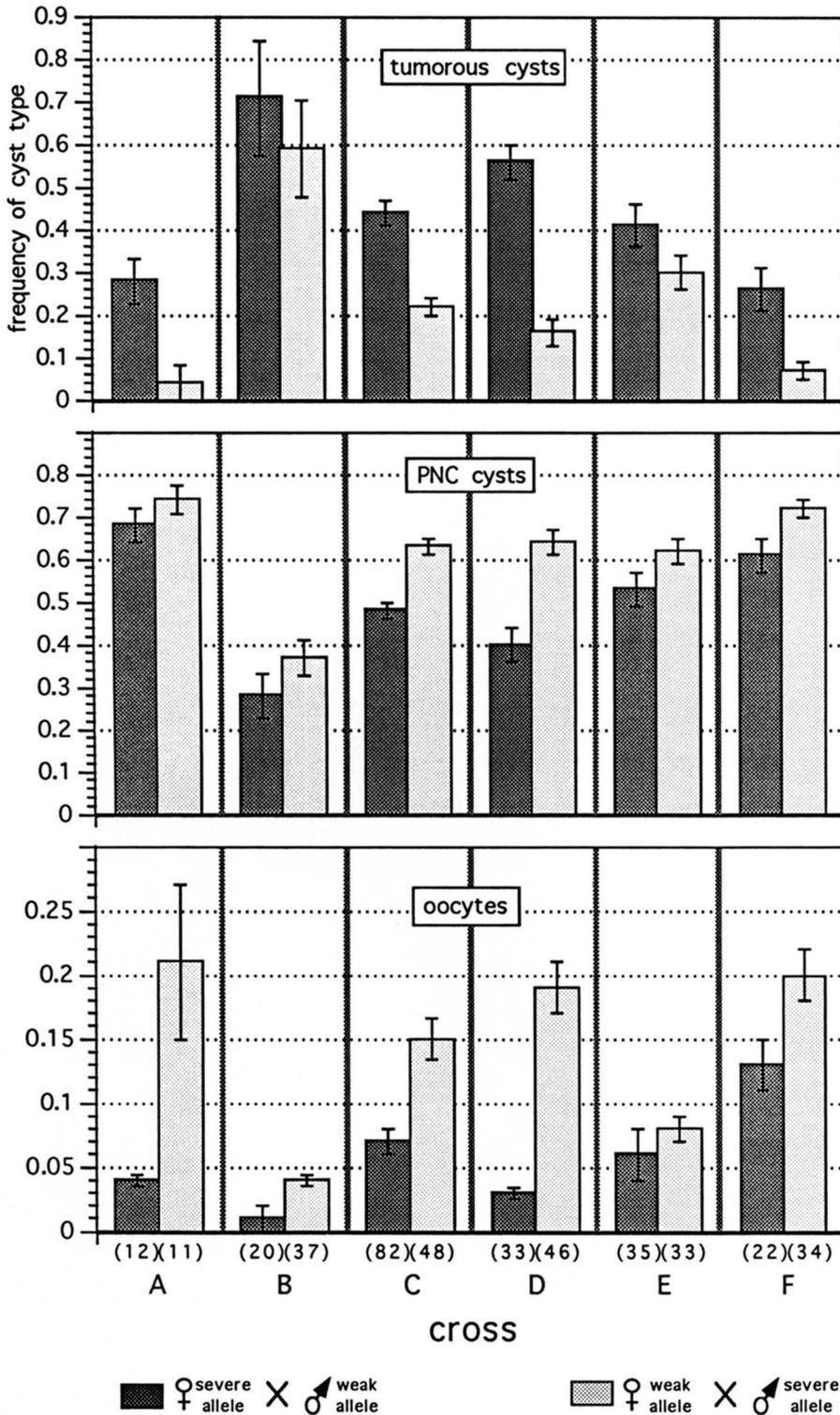


FIGURE 7.—Reciprocal cross effects of egg cyst development between severe and mild *otu* alleles. Reciprocal crosses were done between the severe and mild *otuPΔ* alleles to examine the possibility of a maternal *otu* contribution. The differentiated class alleles (either *otu<sup>PΔ2</sup>* or *otu<sup>PΔ5</sup>*) were crossed to the quiescent class alleles (either *otu<sup>PΔ4</sup>* or *otu<sup>PΔ6</sup>*) in two directions: (1) with the DIF allele maternally contributed and (2) with the DIF allele paternally contributed (described in MATERIALS AND METHODS). The ovaries of the *otu* mutant daughters were examined. Each graph represents reciprocal cross differences found for the frequency of different cyst types in the mutant ovaries. For each set of reciprocal crosses, the daughters were genotypically identical for the X chromosome, but were derived from mothers of different genotypes relative to *otu*. For each mutant ovary, individual egg cysts were categorized and counted. *oocytes*, mature egg cysts containing an oocyte and yolk deposition; *PNC cysts*, egg cysts lacking oocytes but containing cells with large polytene nuclei; *tumorous cysts*, egg cysts of the oncogenic class. A–F represents different sets of reciprocal crosses between two *otuPΔ* alleles. A to D are the results of four independent experiments done with *otu<sup>PΔ4</sup>* and *otu<sup>PΔ2</sup>*; E and F represent two independent experiments with reciprocal crosses between *otu<sup>PΔ5</sup>* and *otu<sup>PΔ4</sup>* (E) and *otu<sup>PΔ5</sup>*-*otu<sup>PΔ6</sup>* (F). Error bars represent the standard error of the mean derived from the original data. Numbers in parentheses indicate the number of ovaries examined. The numerical data used in this figure are found in APPENDIX B.

The *otuPΔ* lesions used in our study are deletions of the promoter region and/or coding sequences of *otu*. Molecular characterization of these alleles demonstrates that they act to reduce or eliminate levels of *otu* transcript and are unlikely to produce a defective polypeptide that may have novel interactions and activity. Therefore, heteroallelic combinations between *otuPΔ* mutations of different classes will produce intermediate levels of *otu* activity relative to the homozygotes. We demonstrate that these intermediate lev-

els of *otu* product result in intermediate mutant phenotypes. These results are in agreement with the conclusions of KING and RILEY (1988). While these data do not preclude the possibility that the two *otu* protein isoforms may have distinct functions, they indicate that the isoform switch is not sufficient for proper oogenesis. Instead, one or both isoforms are required in a dosage-dependent manner at different developmental stages.

**The maternal *otu* RNA contribution affects the**

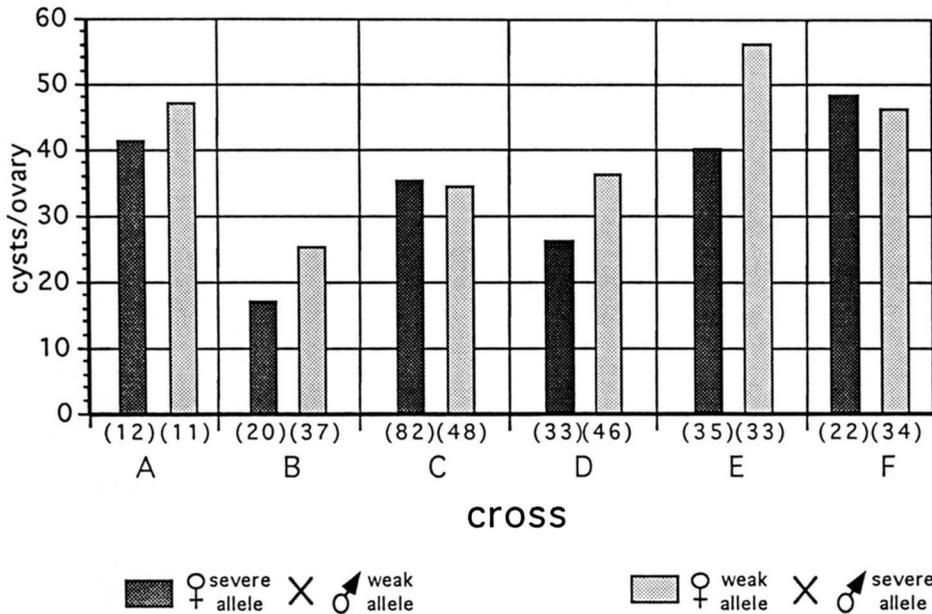


FIGURE 8.—Reciprocal cross effects on the numbers of cysts per ovary. In the same set of crosses described in Figure 7, the mutant ovaries were examined for the numbers of egg cysts present. The numbers of egg cysts per ovary varied from cross to cross in a manner independent of the direction of the cross. Symbols are as described in Figure 7. The numerical data used in this figure are found in APPENDIX B.

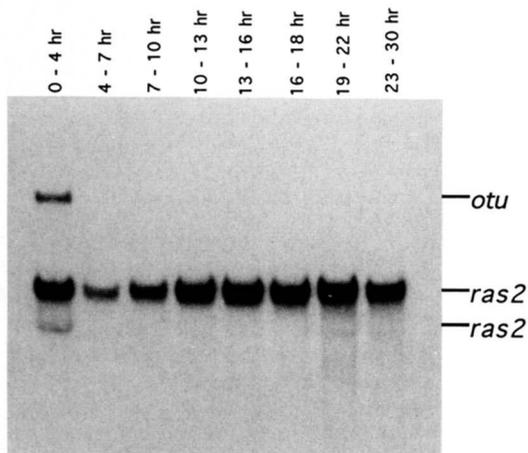


FIGURE 9.—Northern analysis of embryonic RNA. Poly(A<sup>+</sup>) RNA was isolated from various stages of embryonic development. Samples of 10  $\mu$ g of RNA were electrophoresed on a 1.5% agarose/formaldehyde gel. The probe used for the Northern blot as a <sup>32</sup>P-labeled *otu* cDNA described in STEINHAUER, WALSH and KALFAYAN 1989. The filter was reprobred with a DNA fragment containing the *Drosophila ras2* gene (MOZER *et al.* 1985) which serves as a control for the amount of RNA loaded per lane. The numbers at the top refer to the hours of development after embryo collection. The position of migration of *otu* and *ras* mRNA are shown at the right.

**adult ovarian phenotype:** Reciprocal cross experiments suggest that *otu* has an early maternal function. We find that the severity of the *otu* ovarian phenotype arising from different combinations of *otu* alleles is affected by which allele was carried by the mother. The *otu*<sup>-</sup> daughters have a more severe phenotype if their mothers carried the more severe *otu* allele than genotypically identical females derived from mothers that were heterozygous for a more mild *otu* mutation.

The reciprocal cross experiments are complicated by the fact that *otu* is essential for oogenesis thereby requiring that the experimental mothers carry at least

one copy of *otu*<sup>+</sup>. Because of this, we were limited to comparing relatively small differences in the maternal contributions of *otu* from *otu*<sup>null</sup>/*otu*<sup>+</sup> mothers (50% wild-type *otu* activity) versus *otu*<sup>diff</sup>/*otu*<sup>+</sup> mothers (50%–100% of *otu* activity). A second difficulty arises from the variability of the hypomorphic *otu* mutant phenotype, which is affected by environmental conditions such as crowding, age and probably genetic background. Nevertheless, we believe that the reciprocal cross differences reflect variation in the maternal contribution of *otu* for the following reasons. (1) When environmental conditions were kept constant for each set of matings, a consistent pattern of reciprocal effects was obtained for three different allelic combinations, each with different genetic backgrounds. (2) The genetic composition of the progeny from each set of reciprocal crosses are equivalent, *i.e.*, the progeny inherit the same set of parental chromosomes regardless of the direction of the cross. Background differences would only be relevant in the mothers, which itself would suggest a maternal effect on *otu* functions. (3) The reciprocal cross effects were reproducible in four independent experiments with the same two *otu* alleles. These results demonstrate that ovarian morphology is sensitive to relatively small changes in the maternal *otu* levels. Consistent with a maternal function for *otu* is the presence of *otu* transcript during the first 4 hr of embryonic development.

The need for a maternal contribution of *otu* activity suggests an early role for *otu* in pole cell development. Perhaps *otu* acts in parallel with the female-sterile gene *ovo*, which has been shown to be zygotically required for pole cell viability and proliferation (OLIVER, PAULI and MAHOWALD 1990). Alternatively, the *otu* gene may have an early function in the female germline which is phenotypically manifested later in oogenesis.

To examine the temporal requirement for *otu* activity, we are examining pole cell development and larval ovarian morphology in females mutant for the most extreme alleles of *otu*.

STORTO and KING (1987) examined reciprocal cross effects between 23 different mutant combinations and found only two alleles that showed reciprocal cross differences. In these cases, results were consistent with our data, as mothers carrying the more severe allele gave rise to more severely affected daughters. The lack of other examples of reciprocal differences in their study could be due to the use of molecularly uncharacterized EMS-induced mutations. It is possible, if not likely, that interallelic interactions might mask the effects of a maternal contribution. In this regard, even the most severe of the *otu* alleles examined in their study showed interallelic complementation patterns suggestive of functional interactions (KING *et al.* 1986; STORTO and KING 1987; KING and STORTO 1988).

**The *otu* phenotype is variable, ranging from quiescent to oncogenic:** We determined the null phenotype of *otu* by examining in detail the ovarian phenotype of *otu*<sup>PΔ1</sup> homozygotes. The *otu*<sup>PΔ1</sup> mutation deletes the entire *otu* coding region and must be a null mutation. Surprisingly, flies homozygous or hemizygous for this allele often contain tumorous egg cysts (depending on growth conditions) and even occasionally PNC cysts. Therefore, even in the absence of zygotic *otu* product, the female oogonia survive and enter the oogenic developmental pathway. Similar results are obtained with the probable *otu* null allele, *otu*<sup>17</sup>. This mutation is a deletion of a portion of the *otu* coding region that includes the alternatively spliced exons and polyadenylation site (MULLIGAN, MOHLER and KALFAYAN 1988; COMER, SEARLES and KALFAYAN 1992). Northern blot analysis fail to detect a transcript from *otu*<sup>17</sup> homozygotes (COMER, SEARLES and KALFAYAN 1992). Yet, like *otu*<sup>PΔ1</sup>, *otu*<sup>17</sup> mutant ovaries are observed to contain tumorous egg cysts (Figure 5) (KING *et al.* 1986).

The fact that the complete absence of *otu* function produces tumorous and even more mature egg cysts demonstrates that germ cells can survive and proliferate in the absence of zygotic *otu* gene expression. However, our data also indicates that zygotic *otu* activity is important for early oogenesis since a substantial fraction of *otu*<sup>PΔ1</sup> and *otu*<sup>17</sup> mutant ovarioles lack egg cysts. These observations suggest that the *otu* null phenotype is variable between the quiescent and oncogenic phenotypic classes, although occasionally a more mature cyst can develop.

In light of the variable phenotype of *otu* null mutations, we need to re-examine a portion of the dosage hypothesis of STORTO and KING (1987). In this model, the quiescent and tumorous classes of mutant ovarioles result because different levels of *otu* activity are needed during separate developmental periods. This is not consistent with our observation that even in the absence of zygotic *otu* product, tumorous and PNC cysts can develop. A further complication arises from the observation that different null alleles (as deter-

mined by molecular criteria) can differ in the severity of their mutant phenotype. For example, both *otu*<sup>PΔ1</sup> and *otu*<sup>PΔ4</sup> are associated with large deletions within the *otu* locus. Yet *otu*<sup>PΔ4</sup> homozygotes have a more severe average mutant phenotype than *otu*<sup>PΔ1</sup>, rarely if ever producing egg cysts. What accounts for the difference in the severity of these two alleles?

One explanation is that the *otu*<sup>PΔ4</sup> allele may produce an altered product that is detrimental to the development of the egg cysts, perhaps disrupting proliferation of the germline or reducing the viability of germ cells. In this case *otu*<sup>PΔ4</sup> would lead to a more severe phenotype than a null mutation. We believe this model to be unlikely. Genetic studies demonstrate that *otu*<sup>PΔ4</sup> hemizygous ovaries (*otu*<sup>PΔ4</sup>/*otu*<sup>PΔ1</sup>) do not have a more severe mutant phenotype than homozygous null mutations (*otu*<sup>PΔ1</sup>/*otu*<sup>PΔ1</sup>), (APPENDIX A), as would be expected if *otu*<sup>PΔ4</sup> was "poisoning" the oogenic pathway. In addition, no transcript of any size was detected in *otu*<sup>PΔ4</sup> homozygotes from the *otu* region.

An alternative possibility is that the *otu* null phenotype is sensitive to genetic background. Perhaps the *otu* activity is partially redundant. If genes exist that have overlapping functions with *otu*, then the severity of the *otu* mutant phenotype may depend not only on the absolute amount of *otu* activity present, but also on the relative activities of these other genes. Two candidates for genes encoding *otu*-related functions are *bag of marbles* (*bam*) and *ovo*. The predicted *bam* polypeptide has some sequence similarity to a portion of the *otu* product (MCKEARIN and SPRADLING 1990). Mutations in *bam* give ovarian phenotypes similar to *otu*, including the formation of tumorous cysts. Mutations in the *ovo* gene also give rise to a series of phenotypes that are comparable to *otu* mutations (BUSSON *et al.* 1983; OLIVER, PERRIMON and MAHOWALD 1987; MÈVEL-NINIO, MARIOL and GANS 1989; OLIVER, PAULI and MAHOWALD 1990). These include classes of mutations similar to the quiescent, oncogenic, and differentiated ovariole types seen with different *otu* alleles.

Another explanation is suggested by the finding that *otu* has a maternal effect. It is possible that the *otu* maternal contribution occasionally compensates for the absence or reduction of zygotic *otu* activity to allow early stages of oogenesis to occur. The severity of the phenotype of a null *otu* allele would therefore depend on the amount of maternal *otu* product present, the level of which might be influenced by environmental factors and genetic background. In this case, the quiescent and tumorous states may reflect different early requirements for *otu* activity as proposed by the dosage hypothesis, with the *otu* levels determined by both maternal and zygotic contributions.

To address these questions, we are investigating whether *otu* has a role in the embryonic development of the female germline and are examining how *otu* interacts with other genes that regulate oogenesis.

We would like to thank JAN PETTUS for skilled technical assistance and JERRY BEACH for dependable culture media. We appreci-

ate the *otu* clones and information provided by LILLIE SEARLES, ALLEN COMER and GEORGETTE SASS. We thank JOE FRANKEL, WAYNE JOHNSON, KEVIN COOK and KIM COOK for helpful criticism of this manuscript. A special thanks to J. DAWSON MOHLER for the guidance, discussion and inspiration essential for this project. This work was supported by a National Institutes of Health grant (GM45843) and American Cancer Society Institutional Research Seed Grant to R.N.N. and a Basil O'Connor Starter Scholar Research Award from March of Dimes Defects Foundation to P.K.G. (5-FY91-0523).

## LITERATURE CITED

- AVIV, H., and LEDER, 1972 Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Natl. Acad. Sci. USA* **69**: 1408-1412.
- BISHOP, D. L., and R. C. KING, 1984 An ultrastructural study of ovarian development in the *otu7* mutant of *Drosophila melanogaster*. *J. Cell Sci.* **67**: 87-119.
- BUSSON, D., GANS, K. KOMITOPOULOU and M. MASSON, 1983 Genetic analysis of three dominant female sterile mutations located on the X-chromosome of *Drosophila melanogaster*. *Genetics* **105**: 309-325.
- CHAMPE, M.A., and C. D. LAIRD, 1989 Nucleotide sequence of a cDNA from the putative ovarian tumor locus of *Drosophila melanogaster*. *Nucleic Acids Res.* **17**: 3304.
- COMER, A. R., L. L. SEARLES, and L. J. KALFAYAN, 1992 Identification of a genomic DNA fragment containing the *Drosophila melanogaster* ovarian tumor gene (*otu*) and localization of regions governing its expression. *Gene* **118**: 171-179.
- GALIGHER, A. E., and E. N. KOZLOFF, 1971 *Essentials of Practical Microtechnique*, Ed. 2. Lea & Febiger, Philadelphia.
- GANS, M., C. AUDIT and M. MASSON, 1975 Isolation and characterization of sex-linked female-sterile mutants in *Drosophila melanogaster*. *Genetics* **81**: 683-704.
- GEYER, P. K., C. SPANA and V. G. CORCES, 1986 On the molecular mechanism of gypsy-induced mutations at the *yellow* locus of *Drosophila melanogaster*. *EMBO J.* **5**: 2657-2662.
- KING, R. C., 1970 *Ovarian Development in Drosophila melanogaster*. Academic Press, New York.
- KING, R. C., and S. F. RILEY, 1982 Ovarian pathologies generated by various alleles of the *otu* locus in *Drosophila melanogaster*. *Dev. Genet.* **3**: 69-89.
- KING, R. C., and P. D. STORTO, 1988 The role of the *otu* gene in *Drosophila* oogenesis. *Bioessays* **8**: 18-24.
- KING, R. C., S. F. RILEY, J. D. CASSIDY, P. E. WHITE, and Y. K. PAIK, 1981 Giant polytene chromosomes from ovaries of a *Drosophila* mutant. *Science* **212**: 441-443.
- KING, R. C., J. D. MOHLER, S. F. RILEY, P. D. STORTO and P. S. NICOLAZZO, 1986 Complementation between alleles at the ovarian tumor (*otu*) locus of *Drosophila melanogaster*. *Dev. Genet.* **7**: 1-20.
- LINDSLEY, D., and G. ZIMM, 1992 *The Genome of Drosophila melanogaster*. Academic Press, New York.
- MAHOWALD, A. P., and M. P. KAMBYSELLIS, 1980 Oogenesis, pp. 141-224 in *Genetics and Biology of Drosophila*, Vol. 2, edited by M. ASHBURNER and T. R. F. WRIGHT Academic Press, New York.
- MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
- MCKEARIN, D. M., and A. C. SPRADLING, 1990 *bag-of-marbles*: a *Drosophila* gene required to initiate both male and female gametogenesis. *Genes Dev.* **4**: 2242-2251.
- MÉVEL-NINIO, M., M.-C. MARIOL and M. GANS, 1989 Mobilization of the *gypsy* and  *copia*  retrotransposons in *Drosophila melanogaster* induces reversion of the *ovo<sup>D</sup>* dominant female-sterile mutations: molecular analysis of revertant alleles. *EMBO J.* **8**: 1549-1558.
- MOHLER, J. D., and A. CARROLL, 1984 Sex-linked female-sterile mutations. *Drosophila Inform. Serv.* **60**: 236-241.
- MOZER, B., R. MARLOR, S. PARKHURST and V. CORCES, 1985 Characterization and developmental expression of a *Drosophila ras* oncogene. *Mol. Cell. Biol.* **5**: 885-889.
- MULLIGAN, P. K., J. D. MOHLER and L. J. KALFAYAN, 1988 Molecular localization and developmental expression of the *otu* locus of *Drosophila melanogaster*. *Mol. Cell. Biol.* **8**: 1481-1488.
- NAGOSHI, R. N., M. MCKEOWN, K. C. BURTIS, J. M. BELOTE and B. S. BAKER, 1988 The control of alternative splicing at genes regulating sexual differentiation in *D. melanogaster*. *Cell* **53**: 229-236.
- O'CONNELL, P., and M. ROSBASH, 1984 Sequence, structure and codon preference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Res.* **12**: 5495-5514.
- OLIVER, B., D. PAULI and A. P. MAHOWALD, 1990 Genetic evidence that the *ovo* locus is involved in *Drosophila* germ line sex determination. *Genetics* **125**: 535-550.
- OLIVER, B., N. PERRIMON and A. P. MAHOWALD, 1987 The *ovo* locus is required for sex-specific germline maintenance in *Drosophila*. *Genes Dev.* **1**: 913-923.
- PARKS, S., and A. SPRADLING, 1987 Spatially regulated expression of chorion genes during *Drosophila* oogenesis. *Genes Dev.* **1**: 497-509.
- PAULI, D., and A. P. MAHOWALD, 1990 Germ-line sex determination in *Drosophila melanogaster*. *Trends Genet.* **6**: 259-264.
- PERRIMON, N., and M. GANS, 1983 Clonal analysis of tissue specificity of recessive female-sterile mutations of *Drosophila melanogaster* using a dominant female-sterile mutation *Fs(1)K1237*. *Dev. Biol.* **100**: 365-373.
- PERRIMON, N., J. D. MOHLER, L. ENGSTROM and A. P. MAHOWALD, 1986 X-linked female-sterile loci in *Drosophila melanogaster*. *Genetics* **113**: 695-712.
- SPRADLING, A. C., and A. P. MAHOWALD, 1979 Identification and genetic localization of mRNAs from ovarian follicle cells of *Drosophila melanogaster*. *Cell* **16**: 589-598.
- STEINHAEUER, W., and L. J. KALFAYAN, 1992 A specific ovarian tumor protein isoform is required for efficient differentiation of germ cells in *Drosophila* oogenesis. *Genes Dev.* **6**: 233-243.
- STEINHAEUER, W., R.C. WALSH, and L. J. KALFAYAN, 1989 Sequence and structure of the *Drosophila melanogaster* ovarian tumor gene and generation of an antibody specific for the ovarian tumor protein. *Mol. Cell. Biol.* **9**: 5726-5732.
- STORTO, P. D., and R. C. KING, 1987 Fertile heteroallelic combinations of mutant alleles of the *otu* locus of *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **196**: 210-221.
- WIESCHAUS, E., C. AUDIT and M. MASSON, 1981 A clonal analysis of the roles of somatic cells and germ line during oogenesis in *Drosophila*. *Dev. Biol.* **88**: 92-103.

Communicating editor: M. J. SIMMONS

## APPENDIX A

The QUI, TUM, PNC and oocyte classes described in Figure 4 and Figure 6 are derived from the distribution of ovarioles originally classified into eight groups (A-H). These are shown in Table 1. A description of these groups and the equations used to convert these data to the QUI-oocyte classifications are described in the MATERIALS AND METHODS.

## APPENDIX B

Table 2 shows the numerical data used to construct Figure 7 and Figure 8.

**TABLE 1**  
**Mutant ovariole phenotype of different *otu* allelic combinations**

Genotype <sup>a</sup>	Ovariole classification <sup>b</sup>								Total ovarioles
	A (QUI)	B (TUM)	C (T + PNC)	D (T + PNC)	E (PNC)	F (PNC + O)	G (O)	H (T + O)	
$\Delta 1/\Delta 1$	0.44	0.53	0.01	0.02	0.00	0.00	0.00	0.00	1140
$\Delta 2/\Delta 2$	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.98	93
$\Delta 3/\Delta 3$	0.00	0.70	0.10	0.00	0.00	0.00	0.00	0.18	212
$\Delta 4/\Delta 4$	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	168
$\Delta 5/\Delta 5$	0.00	0.00	0.00	0.03	0.23	0.00	0.00	0.77	157
$\Delta 6/\Delta 6$	0.49	0.45	0.04	0.02	0.00	0.00	0.00	0.00	98
$\Delta 1/\Delta 6$	0.48	0.24	0.25	0.02	0.00	0.00	0.00	0.00	213
$\Delta 1/\Delta 4$	0.00	0.91	0.09	0.00	0.00	0.00	0.00	0.00	129
$\Delta 2/\Delta 1$	0.00	0.00	0.00	0.00	0.65	0.23	0.12	0.00	335
$\Delta 2/\Delta 3$	0.00	0.00	0.00	0.00	0.13	0.87	0.00	0.00	97
$\Delta 2/\Delta 4$	0.05	0.08	0.01	0.03	0.57	0.24	0.02	0.00	131
$\Delta 2/\Delta 5$	0.00	0.00	0.00	0.00	0.02	0.98	0.00	0.00	156
$\Delta 2/\Delta 6$	0.10	0.00	0.02	0.04	0.40	0.27	0.16	0.00	93
$\Delta 5/\Delta 1$	0.00	0.28	0.16	0.39	0.09	0.05	0.00	0.02	130
$\Delta 5/\Delta 2$	0.00	0.00	0.00	0.00	0.42	0.47	0.11	0.00	46
$\Delta 5/\Delta 3$	0.00	0.00	0.00	0.13	0.26	0.61	0.00	0.00	129
$\Delta 5/\Delta 4$	0.12	0.04	0.06	0.09	0.60	0.08	0.00	0.01	82
$\Delta 5/\Delta 6$	0.17	0.00	0.02	0.04	0.65	0.11	0.00	0.00	157

<sup>a</sup> The numerator designates the *otu* allele carried by the maternally contributed X chromosome, the denominator the paternally contributed *otu* allele.

<sup>b</sup> Ovariole classification is based on the method of KING and RILEY (1982) described in the MATERIALS AND METHODS. The QUI, TUM, PNC and oocyte values found in Figures 4 and 6 are derived from these data as described in the MATERIALS AND METHODS.

**TABLE 2**  
**Distribution of mutant cyst types in different *otu* allelic combinations**

Cross <sup>a</sup>	Genotype <sup>b</sup>	Tumorous <sup>c</sup>	Pseudonurse <sup>c</sup>	Yolky cysts <sup>c</sup>	Total cysts	Total ovaries	Cysts/ovary <sup>d</sup>
A	$\Delta 4/\Delta 2$	0.28 (0.04)	0.68 (0.04)	0.04 (0.01)	488	12	40.7 (6.2)
	$\Delta 2/\Delta 4$	0.04 (0.04)	0.74 (0.07)	0.21 (0.07)	521	11	47.4 (5.6)
B	$\Delta 4/\Delta 2$	0.71 (0.05)	0.28 (0.05)	0.01 (0.01)	336	20	16.8 (1.5)
	$\Delta 2/\Delta 4$	0.59 (0.04)	0.37 (0.04)	0.04 (0.02)	910	37	24.6 (1.6)
C	$\Delta 4/\Delta 2$	0.44 (0.03)	0.48 (0.02)	0.07 (0.01)	2842	82	34.7 (2.2)
	$\Delta 2/\Delta 4$	0.22 (0.02)	0.63 (0.02)	0.15 (0.02)	1644	48	34.3 (2.4)
D	$\Delta 4/\Delta 2$	0.56 (0.04)	0.40 (0.04)	0.03 (0.01)	868	33	26.3 (2.0)
	$\Delta 2/\Delta 4$	0.16 (0.03)	0.64 (0.03)	0.19 (0.02)	1646	46	35.8 (1.7)
E	$\Delta 4/\Delta 5$	0.41 (0.05)	0.53 (0.04)	0.06 (0.02)	1403	35	40.0 (4.8)
	$\Delta 5/\Delta 4$	0.30 (0.04)	0.62 (0.03)	0.08 (0.01)	1846	33	55.9 (3.9)
F	$\Delta 6/\Delta 5$	0.26 (0.05)	0.61 (0.04)	0.13 (0.02)	1045	21	49.8 (5.2)
	$\Delta 5/\Delta 6$	0.07 (0.02)	0.72 (0.02)	0.20 (0.02)	1569	34	46.1 (3.6)

<sup>a</sup> Crosses are the same as that shown in Figure 7 and Figure 8. See MATERIALS AND METHODS for details of the crosses.

<sup>b</sup> The numerator designates the *otu* allele carried by the maternally contributed X chromosome, the denominator the paternally contributed *otu* allele.

<sup>c</sup> Numbers represent the fraction of cysts that are of the designated phenotype.

<sup>c,d</sup> Numbers in parentheses are the standard error of the mean.