Developmental Analysis of the *ovarian tumor* Gene During Drosophila Oogenesis

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ABSTRACT

Severe alleles of the *ovarian tumor* (*otu*) and *ovo* genes result in female sterility in *Drosophila melanogaster*, producing adult ovaries that completely lack egg chambers. We examined the developmental stage in which the agametic phenotype first becomes apparent. Germ cell development in embryos was studied using a strategy that allowed simultaneous labeling of pole cells with the determination of embryonic genotype. We found that ovo^- or otu^- *XX* embryonic germ cells were indistinguishable in number and morphology from those present in wild-type siblings. The effects of the mutations were not consistently manifested in the female germline until pupariation, and there was no evidence that either gene was required for germ cell viability at earlier stages of development. The requirement for *otu* function in the pupal and adult ovary is supported by temperature-shift experiments using a heat-inducible *otu* gene construct. We demonstrate that *otu* activity limited to prepupal stages was not sufficient to support oogenesis, while induction during the pupal and adult periods caused suppression of the *otu* mutant phenotype.

DETAILED studies have been made on the differentiation and behavior of germ cells during development (KING 1970; KING and MOHLER 1975; MAHOWALD and KAMBYSELLIS 1980; SPRADLING 1993). The pole cells are the precursors to the germline. They are initially localized at the posterior pole of the embryo, eventually forming a cluster of 40–60 cells. The pole cells migrate anteriorally during germ band extension, invaginating into the mesoderm where they split into two groups to create the embryonic gonad (UNDERWOOD *et al.* 1980; KOBAYASHI *et al.* 1993). Only a subset of the pole cells (approximately eight) will ultimately form the female germline (SPRADLING 1993). By 16 hr, these cells undergo a series of divisions and are physically demarcated from the rest of the embryonic structures.

During larval stages the ovaries remain small and mostly undifferentiated. They consist of large germ cells (oogonia) and smaller mesodermal cells that give rise to follicle cells and the ovarian sheaths. In the late third instar larval stage, the oogonia proliferate to form the germline stem cell population. During pupariation, the somatic gonad undergoes dramatic morphological changes that will lead to the formation of the adult ovary. A series of elongated tubes (ovarioles) form, each containing approximately two stem cells located at the apical tip. Approximately 10–17 ovarioles make up a single ovary. The development of the somatic ovary, including the formation of ovarioles, does not require the presence of germ cells (FIELDING 1967; ENGELS and PRESTON 1979; NIKI and OKADA 1981; ENGSTROM *et al.* 1982).

Egg chamber development is initiated in the germarium by the asymmetric division of the stem cells, producing a cystoblast and daughter stem cell. The cystoblast undergoes four mitotic divisions characterized by incomplete cytokinesis to produce 16 cystocytes connected by intercellular bridges. One of these cystocytes becomes the oocyte, while the other 15 differentiate into large polyploid nurse cells. At the 16-cystocyte stage, somatically derived follicle cells envelope the syncytium to form the egg chamber. The subsequent maturation of the egg cyst has been divided into 14 stages and occurs continuously throughout the adulthood (KING 1970). Therefore, each ovariole consists of a string of progressively older egg chambers with the youngest cysts in the apical germarial region and more mature chambers near the oviduct.

The *ovarian tumor* (*otu*) gene is required in the germline at several stages during oogenesis. The *otu* mutant alleles are subdivided into three classes based on their predominant ovarian phenotypes (KING *et al.* 1986; KING and STORTO 1988). Quiescent mutations are defective in germ cell viability or proliferation such that the ovaries completely lack egg chambers. Oncogenic *otu* alleles cause the ovarian tumor phenotype in which egg chambers are filled with thousands of mitotically active, apparently undifferentiated, germ cells (KING and RILEY 1982; BISHOP and KING 1984). The least severe *otu* mutant class results in the partial development of the female germline. Egg chambers differentiate to a stage where they contain nurse cells and oocytes but fail to mature into functional eggs. In contrast to these severe effects on oogenesis, males carrying a deletion of the *otu*-coding region are fertile (GEYER *et al.* 1993; SASS *et al.* 1993).

The molecular cloning of the *otu* gene has been pre-

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viously described (MULLIGAN *et al.* 1988; CHAMPE and LAIRD 1989; STEINHAUER *et al.* 1989). A 5.0-kb genomic DNA fragment rescues the female sterility caused by *otu* mutations (COMER *et al.* 1992). Analysis of *otu*-specific cDNAs indicate that two alternatively spliced RNAs are produced that encode *otu* protein isoforms of 98- and 104-kD (STEINHAUER and KALFAYAN 1992). Both the *otu* RNA (PARKS and SPRADLING 1987) and proteins (STEINHAUER and KALFAYAN 1992) are expressed in germ cells of the adult ovary. This tissue localization is consistent with the finding that *otu* expression is required in the germ line, not soma, for oogenesis (WIESCHAUS *et al.* 1981; PERRIMON and GANS 1983). Genetic studies with isoform-specific *otu* alleles suggest that both OTU proteins are active in the germline. Alleles that produce only the 98-kD isoform allow the production of tumorous egg chambers but no further oogenic differentiation (BAE *et al.* 1994; NAGOSHI *et al.* 1995). Furthermore, we have shown that the 104-kD product is required for the differentiation of nurse cells in a *Sxl*-dependent manner (BAE *et al.* 1994). Curiously, however, under certain conditions the 104-kd product is capable of providing all *otu* function required for oogenesis (SASS *et al.* 1995).

The *ovo* gene gives rise to a similar range of mutant phenotypes as *otu* (BUSSON *et al.* 1983; OLIVER *et al.* 1987, 1990; MÈVEL-NINIO *et al.* 1989). The absence of *ovo* function results in the formation of agametic ovaries but has no effect on male gametogenesis (OLIVER *et al.* 1990). Hypomorphic alleles can produce ovarian tumors or late stage arrest of oogenesis (OLIVER *et al.* 1987). Like *otu, ovo* activity is germline-dependent (WIESCHAUS *et al.* 1981; PERRIMON and GANS 1983). The *ovo* locus is molecularly complex with a number of overlapping transcripts and two overlapping functions (MÈVEL-NINIO *et al.* 1991; GARFINKEL *et al.* 1992). Sequence analysis indicates that at least a subset of *ovo* products contain regions with homology to zinc-finger domains (MÈVEL-NINIO *et al.* 1991).

The specific roles of *otu* and *ovo* in oogenesis are not known. It has been suggested that one or both genes play a role in the determination of germline sex, *i.e.*, in establishing the sexual identity of the germ cell before sexual differentiation has occurred (OLIVER *et al.* 1990, 1993, 1994; STEINMANN-ZWICKY 1992; WEI *et al.* 1994). Perhaps the most compelling evidence for this hypothesis comes from genetic studies on the embryonic phenotype of severe *ovo* mutations (OLIVER *et al.* 1987, 1990). In these experiments it was suggested that the absence of zygotic *ovo* activity resulted in the loss of female germ cells during embryogenesis. This observation strongly implicates the involvement of *ovo* during the initial stages of germline sex determination. Alternatively, it may be that *otu* and *ovo* control processes specific to oogenesis (KING and STORTO 1988; NAGOSHI *et al.* 1995). As such, both genes would be regarded as responding to, rather than being responsible for, the sexual identity of the germline.

In this study, we undertook a set of experiments to determine the possible role of *otu* in sex-specific germ cell viability, proliferation, and differentiation. We were particularly interested in determining the developmental stages during which *otu* function is required. Our results demonstrate that *otu* mutations do not have a detectable effect on germ cell proliferation or differentiation until the pupal period. These observations are consistent with temperature shift experiments in which *otu* activity was induced at various developmental stages. Expression during the pupal and adult periods was sufficient to suppress the *otu* mutant phenotype. Our studies on *ovo* mutants gave results similar to that described for *otu*. We found no evidence that *ovo* mutations caused embryonic or larval germ cell death. In fact, we confirm that *ovo*$^-$ germ cells can accumulate in the germarial region of adult agametic ovaries. This forces a reevaluation of the possible roles of *ovo* in oogenesis and germline sex determination.

MATERIALS AND METHODS

Fly stocks: The *otu* alleles used in this study have been extensively characterized and their phenotypes summarized in Table 1 (KING and RILEY 1982; KING *et al.* 1986; STORTO and KING 1987). The *otu*^{2} and *otu*^{10} alleles are the most severe EMS-induced mutations and have mutant phenotypes virtually identical to a deletion of the *otu* gene (GEYER *et al.* 1993; SASS *et al.* 1993). The *ovo*^{D1rS1} and *lzl*^{G} alleles are two of the most severe *ovo* alleles and were previously used to demonstrate embryonic germ cell death in females (OLIVER *et al.* 1987, 1990). The *y w ovo*^{D1rS1} chromosome was derived by recombination from an *ovo*$^{D1rS1\ v24}$ chromosome. *FM7z* is an *FM7* balancer chromosome carrying a minigene where the *fushi tarazu* promoter is fused to the bacterial *lacZ* gene (obtained from R. FINKELSTEIN). The germline-specific *BC69* enhancer trap line was obtained from F. LASKI. This enhancer trap line is associated with a female sterile mutation that is allelic to *vasa* (F. LASKI, personal communication). Expression of *BC69* mimics that of the *vasa* gene. Descriptions of other mutations and balancer chromosomes used in this study are found in LINDSLEY and ZIMM (1992). Flies were raised on a standard cornmeal, molasses, yeast, agar media containing propionic acid as a mold inhibitor and supplemented with live yeast.

Transgene constructs: The *hs-otu* minigene was constructed by cloning a 4-kb *Hpa*I *otu* genomic fragment between the Drosophila *hsp70* promoter and the polyadenylation sequences of the α-tubulin gene. This fusion gene was subsequently cloned into *pDM23*, a transformation vector carrying the xanthine dehydrogenase gene (*rosy*) as a selectable transformation marker. This fragment of *otu* contains genomic sequences starting 64 nucleotides downstream of the transcription start site and ends 139 nucleotides downstream of the translation stop codon. Germline transformation was carried out as previously described (RUBIN and SPRADLING 1982). The host strain used in transformation was *y ac/y ac; ry*^{506}/*ry*^{506}. Germline transformants were identified by the rescue of the *rosy* mutant phenotype. Several transformed lines were isolated and the chromosomal locations of the transposon was determined by standard genetic crosses.

The *otu-lacZ* construct was made by blunt end ligation of a

fragment containing *otu* sequences between −1027 and +63 relative to the start site of transcription into the *Bam*HI site of *pCaSpeR AUG-β-gal* (THUMMEL *et al.* 1988). Germline transformation of the *otu-lacZ* fusion gene was carried out as described above for the *hs-otu* minigene. Insertions of the *otu-lacZ* transgene were obtained on every chromosome. In these experiments, transgene insertions on the second or third chromosome were crossed into the appropriate genetic background for use as a marker for the presence of germ cells.

β-galactosidase and DAPI staining of whole mount ovaries: Larval or adult ovaries were dissected in phosphate-buffered saline (PBS), and the tissues were then treated according to established staining procedures (ASHBURNER 1989).

Immunohistochemistry: Embryos were collected at 25° for 8–10 hr overnight on 3% agar apple juice plates supplemented with fresh yeast paste. The embryos were dechorionated using 50% commercial bleach for 5 min, rinsed several times with water, and transferred to corex tubes for fixation in a 1:1 mix of 4% paraformaldehyde (in PBS) and heptane. Visualization of the VASA protein was accomplished by incubating with a 1:500 dilution of the monoclonal (IgM class) antibody line no. 46F11 (HAY *et al.* 1988) kindly provided by IRA CLARK. β-galactosidase was detected using a monoclonal IgG antibody (Promega Co.) diluted 1:1000. Secondary antibodies conjugated with horseradish peroxidase and directed against mouse IgG (anti-βGAL) or mouse IgM (anti-VASA) were used to label *in situ* preparations.

Pole cell counts: Embryos were collected as mentioned above. The time of incubation was optimized to recover a high percentage of embryos from stages 7–11 [stages according to CAMPOS-ORTEGA and HARTENSTEIN (1985)]. Embryos were labeled with anti-VASA (HAY *et al.* 1988) and anti-βgal (mouse IgG-Promega), dehydrated, and mounted in 1:1 permount/xylene. The pole cells were counted using an Olympus Vanox microscope with bright field and Nomarski optics. The data in Figure 2 are pooled data from embryos at stages 7–11. During these stages, the pole cells migrate, become internalized, and form the embryonic gonad.

Temperature shift experiments: Eggs from the cross otu^{10}/*FM0* × $otu^{P\Delta 1}$/*Y; hs-otu/CyO* were collected every 24 hr at 25°. Each 24-hr collection was transferred to vials and subject to different temperature conditions. The heat shock regimen consisted of a daily 1-hr 37° treatment in a dry air incubator followed by overnight incubations at 25°. The nonheat shock regimen consisted of constant growth at 18°. The following culture conditions were tested. Continuous incubation at 18° was appplied for 18° control. Embryonic heat shock (E): the heat shock regimen was applied during 24–72 hr after collection followed by culturing at 18° for the rest of development. At the time of the shift, the majority of flies were in the embryonic or first instar larval stages. Embryonic to larval heat shock (E-L): the heat shock regimen was applied until the first pupae were observed followed by incubation at 18° for the rest of development. Embryonic to pupa (E-P): the heat shock regimen was applied until the first adults eclosed followed by incubating at 18° for the rest of development. Embryo to adult heat shock (E-A): the heat shock regimen was applied throughout development. Larval-specific heat shock (L): animals were cultured at 18° for 48–72 hr followed by exposured to the heat shock regimen until the first pupae formed. The cultures were then shifted to 18° until dissection. Pupal-specific heat shock (P): the heat shock regimen was applied only during the pupal period. Otherwise, the animals were cultured at 18°. Pupal to adult heat shock (P-A): animals were cultured at 18° until the first pupae formed followed by the heat shock regimen until dissection. Adult-specific heat shock (A): flies were kept at 18° until the first day of eclosion and were exposed to the heat shock regimen until dissection. Females cultured as adults under the heat shock regimen (E-A, P-A) were aged 4–6 days before dissection, except for the adult-specific heat shock experiment where adult females were cultured for 7 days. Females cultured as adults at 18° were aged 8–12 days before dissection. The dissected ovaries were examined by Feulgen staining as described in GEYER *et al.* (1993).

RESULTS

Zygotic *otu* activity is not required for germ cell proliferation or viability in embryos: The most severe *otu* alleles produce ovaries that are devoid of egg chambers (KING *et al.* 1986). We investigated when in development this agametic phenotype first becomes apparent. We were particularly interested in determining whether the *otu* gene was needed for the accumulation of embryonic germ cells, as was reported for the *ovo* gene (OLIVER *et al.* 1987, 1990). If this were the case, it would suggest that *otu* may be required for the initial stages of germline sexual differentiation.

In these studies, we examined otu^- embryos generated by mating otu^-/*FM7z* females with otu^-/*Y* males. *FM7z* is an otu^+ balancer chromosome carrying a construct in which the bacterial *lacZ* gene is controlled by the Drosophila *fushi tarazu* promoter. This construct expresses β-galactosidase in a segment-specific pattern throughout most of embryogenesis (Figure 1A), thereby unambiguously identifying the otu^+ (otu^-/*FM7z* and *FM7z/Y*) embryos. To identify the embryonic germ cells, we used an antibody specific to the germline-dependent vasa protein in *in situ* preparations (HAY *et al.* 1988; LASKO and ASHBURNER 1990; WEI *et al.* 1991) (Figure 1B).

There are two possible outcomes from this experiment. If *otu* activity is required in female embryonic germ cells, then there should be a significant reduction in the number of otu^-/otu^- pole cells relative to their otu^-/*Y* male or otu^+ siblings. This would result in a bimodal distribution of pole cell numbers among the non-*FM7z* (otu^-/otu^- and otu^-/*Y*) embryos in contrast to the single peak expected from the otu^+ (otu^-/*FM7z* and *FM7z/Y*) population. Alternatively, if *otu* does not affect embryonic *XX* germ cell numbers, then there should be no differences in pole cell numbers between otu^-/otu^-, otu^-/*Y*, and otu^+ embryos.

Two *otu* alleles were examined, otu^{10} and otu^2. These are the among the most severe *otu* lesions (KING *et al.* 1986), producing ovarian phenotypes as severe as that obtained with deletions of the *otu* gene (GEYER *et al.* 1993). The results for otu^{10} and otu^2 were identical, so only the combined data are presented (Figure 2A). We found no differences in the numbers or morphology of *otu* mutant embryonic germ cells compared to wild-type during stages when the pole cells were invaginating and forming the embryonic gonads [embryonic stages 7–11 as described by CAMPOS-ORTEGA and HARTENSTEIN (1985)]. In addition, a comparison between *otu* mutant and otu^+ embryos demonstrated virtually identi-

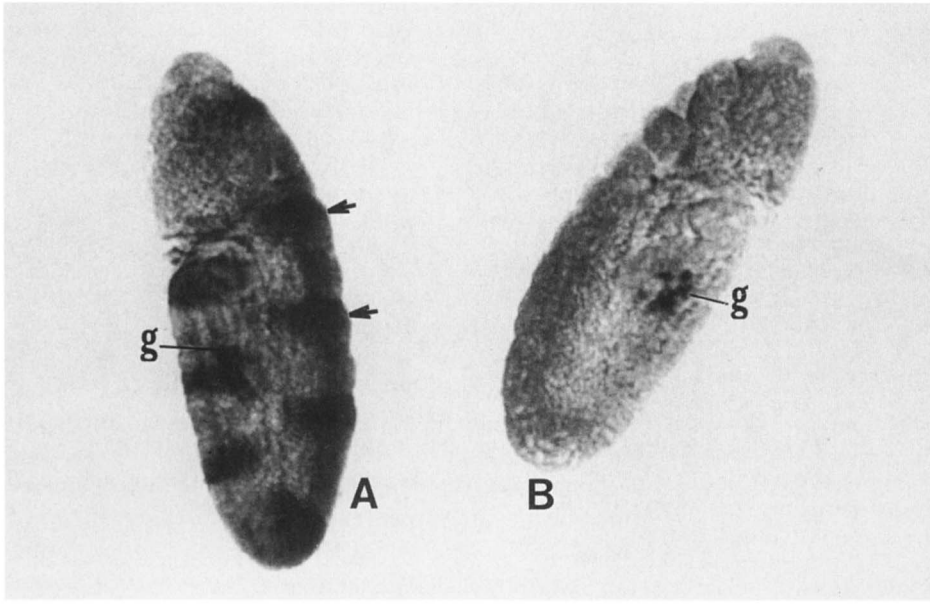

FIGURE 1.—VASA-specific and β-galactosidase-specific antibody labeling of embryos. Embryos were derived from a cross between otu^- (or ovo^-)/*FM7z* × otu^- (or ovo^-)/*Y*. The *FM7z* chromosome is an ovo^+, otu^+ balancer carrying the bacterial *lacZ* gene fused to the *fushi tarazu (ftz)* promoter, *ftz(lacZ)*. Wild-type embryos (A) will carry *ftz(lacZ)* and therefore express β-galactosidase in a segment-specific pattern, as seen by antibody labeling (arrow). The absence of the *ftz(lacZ)* stripe pattern indicates a mutant embryo (B). Embryonic germ cells (g) are specifically recognized by a VASA-specific antibody. The *in situ* localization of antibodies was visualized by horse radish peroxidase staining.

cal distribution profiles. These results indicate that reductions in zygotic *otu* activity sufficient to cause agametic adult ovaries do not affect the proliferation or viability of the embryonic germline.

***ovo* activity is not essential for the development of the embryonic germline:** We wanted to compare the results of the *otu* experiments with a mutation believed to affect pole cell viability. It was previously reported that the ovo^{D1rS1} mutation caused a significant reduction in the number of germ cells in female embryos, leading to the suggestion that *ovo* may be required for the initial steps in germline sex determination (OLIVER *et al.* 1987, 1990). This *ovo* allele is amorphic, producing agametic ovaries while having no effect in males (OLIVER *et al.* 1987). We repeated these studies using the same methodology described for otu^{10} and otu^2.

To our surprise we saw no evidence of embryonic germ cell loss caused by ovo^{D1rS1} (Figure 2B). The distribution curve obtained for the combined population of ovo^-/ovo^- and ovo^-/Y embryos displayed a single peak that was virtually identical to that derived from ovo^+ siblings. As with *otu*, these observations were based primarily on embryos in stages 7–11. Although these specimens were generally more mature than those in which

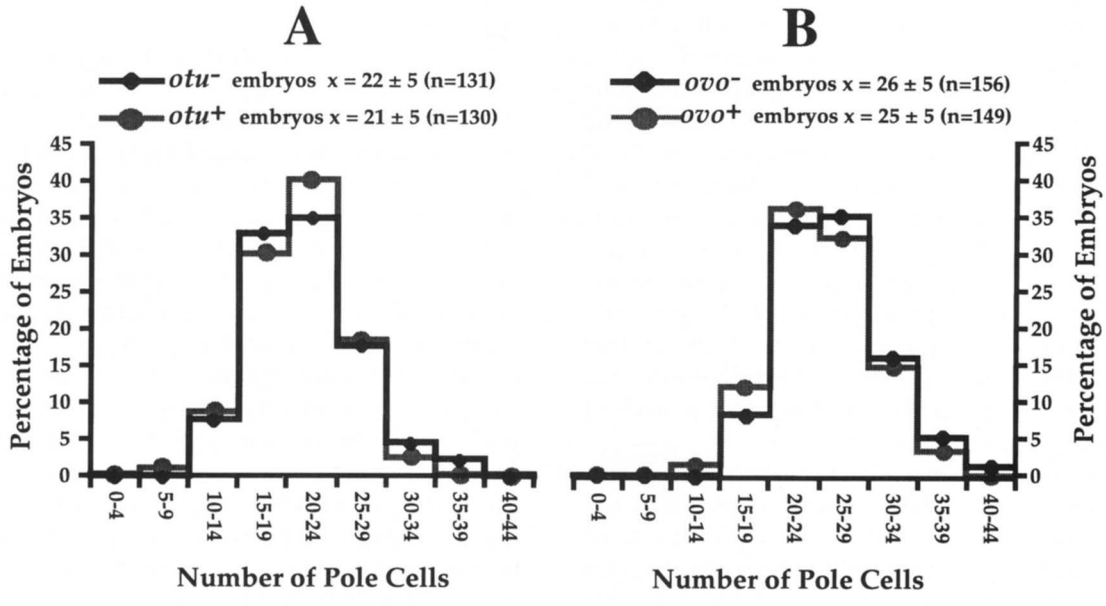

FIGURE 2.—Germ cell numbers in *otu* and *ovo* mutant embryos. Agametic alleles of *otu* and *ovo* were examined for their effect on embryonic germ cells during stages 8–14 of embryogenesis. Germ cells and embryos were labeled as described in Figure 1. Each point represents the percentage of embryos that contain a given range of germ cells. A represents pooled data from *otu* mutant embryos derived from a cross of sn^3 otu^{10} *v f*/*FM7z* × sn^3 otu^{10} *v f*/*Y* and sn^3 otu^2 *v f*/*FM7z* × sn^3 otu^2 *v f*/*Y*. B represents *ovo* mutant embryos from cross of *y w* ovo^{D1rS1}/*FM7z* × *y w* ovo^{D1rS1}/*Y*. n, number of embryos examined.

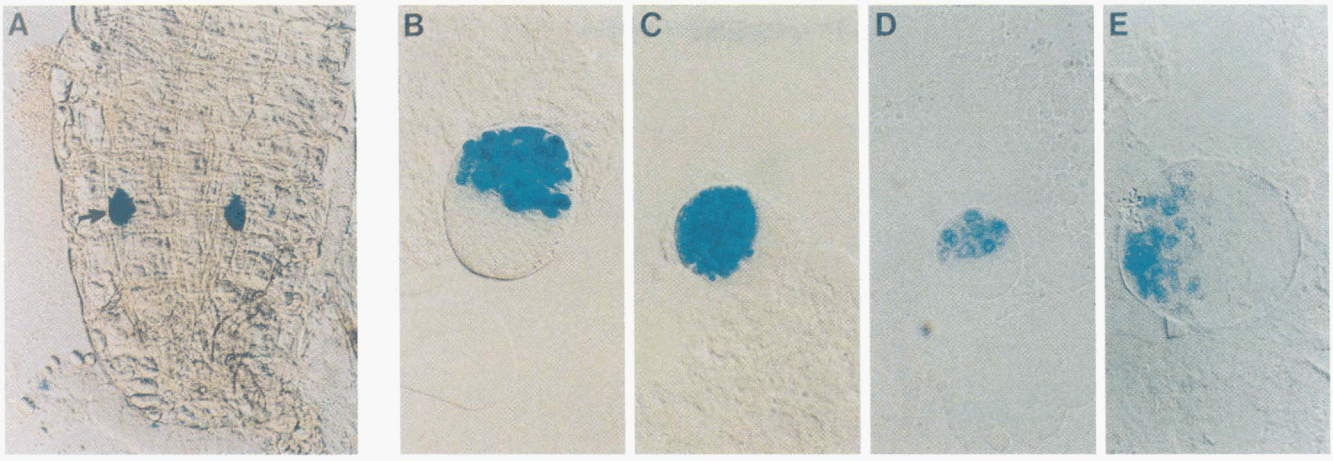

FIGURE 3.—Morphology of wild-type and mutant larval gonads. The larval germ cells were marked by the expression of β-galactosidase (stained blue) activity from the germline-specific *otu-lacZ* construct. (A) Wild-type ovaries (arrow) from first instar larvae shown using a 10× objective. B–D were photographed using a 40× objective. (B) Wild-type third instar larval ovary. (C) Third instar larval ovary mutant for an agametic *otu* allele (*otu10*). (D) third instar larval ovary mutant for an agametic *ovo* allele (ovo^{D1rS1}). (E)Third instar larval ovary mutant for ovo^{D1rS1} shown at twofold higher magnification than D. Substantial differences in the size of third instar larval ovaries are often seen and do not correlate with the mutant *otu* or *ovo* genotypes.

the loss of ovo^{-} germ cells were reported (approximately stages 4–9) (OLIVER *et al.* 1987, 1990), we would have expected this deficiency to persist throughout subsequent development if it were to account for the adult mutant phenotype. In contrast, our findings suggest that any early reduction in germ cell numbers is either transient or inconsistent. We therefore conclude that like *otu*, zygotic *ovo* activity is not essential for pole cell viability or proliferation during later stages of embryogenesis and that the germline disruptions that lead to the adult agametic phenotype most likely occur postembryonically.

There is no evidence of germ cell loss in *otu* mutant larval gonads: To determine when germ cell arrest occurs in the *otu* and *ovo* mutants, we examined ovarian development in postembryonic stages. To more easily identify germ cells, we constructed an *otu-lacZ* minigene in which 1027 bp of the *otu* promoter region was fused to the *lacZ* gene. Flies carrying this construct expressed β-galactosidase in the gonads throughout postembryonic stages. To test the germline specificity of *otu-lacZ*, we examined progeny from female mutants for the maternal-effect gene *tudor*. These progeny lack a germline but can still develop a somatic gonad (BOSWELL and MAHOWALD 1985). As expected, we found no evidence of *otu-lacZ* expression in the larval or adult gonads of these progeny (data not shown).

Expression from *otu-lacZ* is initially detected throughout wild type first instar larval ovaries (Figure 3A) but becomes delimited to $^{1}/_{2}$–$^{2}/_{3}$ of the ovary by the third instar (Figure 3B). Similar staining patterns were seen using the enhancer trap line associated with *vasa* (*BC69*) (data not shown). This suggests that *otu-lacZ* is probably expressed in most, if not all, larval germ cells. While these patterns of expression for *otu-lacZ* and *BC69* were consistently reproducible, we found considerable variability in ovary size and in the intensity of β-galactosidase staining, even among staged wild-type larvae. We therefore limited our analysis to a comparison of the distribution pattern of β-galactosidase expressing cells in wild-type and mutant animals.

In observations of over 50 larvae, we found that otu^{10} mutant ovaries were morphologically indistinguishable from wildtype (representative gonads are shown in Figure 3, B and C). This indicates that *otu* activity is not essential for the accumulation and distribution of *XX* larval germ cells. While subtle defects would be difficult to detect by this analysis, it is clear that the gross aberrations characteristic of the otu^{10} adult phenotype had not yet occurred.

We also tested the effect of the agametic *ovo* allele, ovo^{D1rS1}, on the larval germ cells. While we consistently detected *ovo* mutant germ cells in third instar larval ovaries, the extent of *otu-lacZ* expression was more variable. Many ovaries showed a near wild type staining pattern, while others contained fewer and more weakly staining germ cells (Figure 3, D and E). Similar results were obtained with the agametic *ovo* heteroallelic combination lzl^{G}/ovo^{D1rS1} (data not shown). The lzl^{G} allele is similar to ovo^{D1rS1} in that it produces agametic adult ovaries when homozygous and was also reported to eliminate *XX* germ cells during embryogenesis (OLIVER *et al.* 1990). The variable expression of *otu-lacZ* in these studies could result because *ovo* mutations so disrupt the development of female germ cells that gene expression is generally and nonspecifically affected. It is also possible that *ovo* has a more specific role in controlling *otu* promoter activity, however, this is inconsistent with the frequent occurrence of wild-type levels and patterns of *otu-lacZ* expression in *ovo* mutants.

Female germ cells can survive and accumulate in pupal and adult ovaries even in the absence of zygotic *otu*

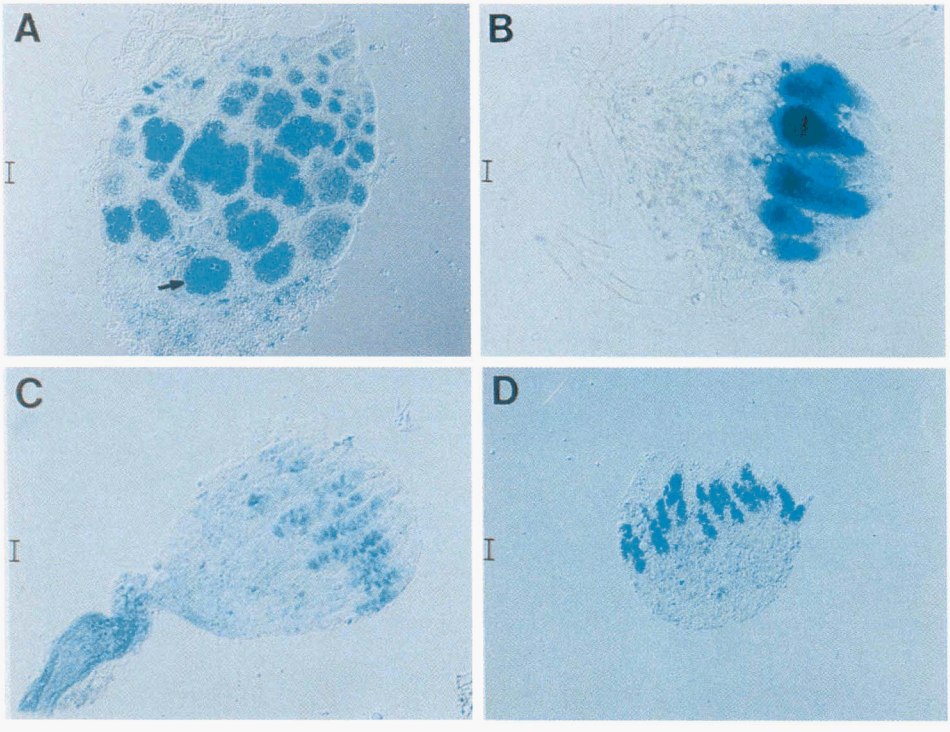

FIGURE 4.—Morphology of wild-type and mutant pupal ovaries. Germ cells are marked by the germline expression of β-galactosidase activity from the *otu-lacZ* construct (A–C) or the *BC69* enhancer trap line (D). (A) Wild-type pupal female gonads. Note egg chambers with nurse cells (arrow). (B) Pupal ovary mutant for otu^{10}. Stained germ cells localized in apical portion of ovarioles. (C) Pupal ovaries mutant for ovo^{D1rS1}. Stained cells distributed in ovarioles. (D) Less mature ovo^{D1rS1} mutant pupal ovary showing a number of stained germ cells distributed in the ovarioles. Horizontal bar is a relative measure of magnification.

or *ovo* activity: In wild-type pupal ovaries, *otu-lacZ* is expressed in the germaria and in the developing egg chambers (Figure 4A). By late pupae, the ovarioles are well defined and individual egg chambers contain prominently stained nurse cells. In contrast, we found that gonads mutant for otu^{10} or ovo^{D1rS1} failed to produce egg chambers and the germ cells that were present did not differentiate. Ovarioles were frequently observed that contained large clusters of germ cells localized in the apical region coincident with the presumptive germaria (Figure 4B). More severely affected ovaries had fewer staining cells that were more randomly dispersed in the ovariole, occasionally forming small clusters (Figure 4C). The absence of pupal egg chambers indicates that the agametic adult phenotype occurs because oogenesis is blocked before cyst formation, rather than from the degeneration of existing chambers. Furthermore, the consistent observation of *otu* or *ovo* mutant germ cells in pupal ovaries demonstrates that neither gene is essential for germline viability before pupariation.

In the adult stage, the mutant ovaries are generally more severely affected with fewer cells expressing germline markers. Whereas wild-type adult ovaries showed substantial *otu-lacZ* activity in all oogenic stages (Figure 5A), ~20% of otu^{10} adult ovaries had no *otu-lacZ* expression. The remaining mutant ovaries had one or more ovarioles that contained a cluster of cells localized at the apical tip (Figure 5B). These mutant cells were small, undifferentiated, and failed to become encysted. They were most likely arrested at or before the stage of cystoblast differentiation and division, when the germline and follicle cells normally interact to form the egg chamber. Identical results were obtained for several different *otu* allele combinations, including a homozygous deletion of the entire *otu*-coding region (data not shown).

Similar results were seen in ovo^{D1rS1} adults or with the agametic *ovo* allele combination, lzl^{G}/ovo^{D1rS1}. Although many mutant ovaries were devoid of detectable germ cells (Figure 5C), a substantial fraction (25–30%) had ovarioles that contained one or more small clusters of germ cells (Figure 5, D and E). On rare occasions, small egg chambers were produced that contained variable numbers of nurse-like cells (Figure 5E). These data indicate that while *ovo* is required for the normal accumulation and differentiation of the female germline, some germ cells can survive into the adult stage in the absence of wild-type *ovo* activity. Similar evidence for ovo^{-} germ cell survival into adult stages has been reported using an *ovo*-specific marker gene (OLIVER *et al.* 1994).

To support the *otu-lacZ* results, analogous studies were done using the germline-specific *BC69* enhancer trap line in larvae, and early- and midstage pupae mutant for *otu* or *ovo* (a midstage ovo^{-} pupal ovary is shown in Figure 4D). The results were identical to that obtained with *otu-lacZ,* demonstrating again that the morphological aberrations associated with *otu* and *ovo* mutations first become apparent during pupariation. Curiously, however, both *otu* and *ovo* mutant late pupal and adult ovaries rarely contained cells expressing *BC69,* even though the *otu-βgal* construct indicated that germ cells are often present in these gonads. It may be that the mutant germ cells are abnormal in their activation of germline-specific enhancers or that they are arrested at a stage when the *BC69* line is not active. In any case,

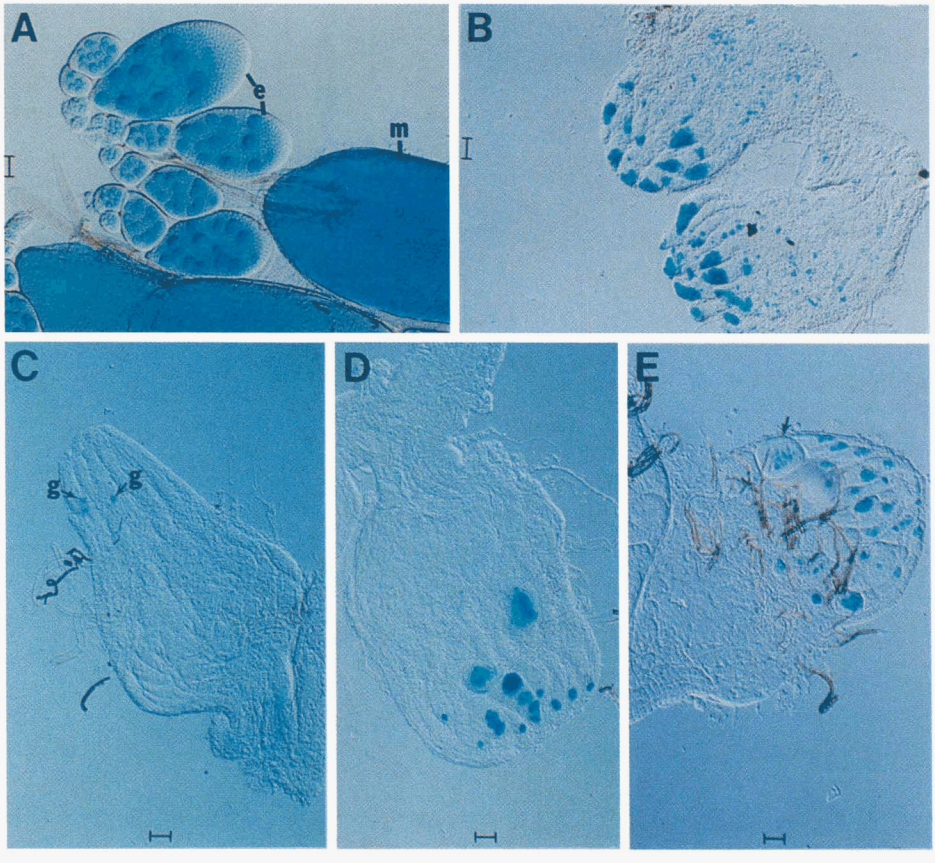

FIGURE 5.–Morphology of wild-type and mutant adult ovaries. Germ cells are marked by the germline expression of β-galactosidase activity from the *otu-lacZ* construct. (A) Wild-type pupal female gonads. (B) Pupal ovary mutant for otu^{10}. (C–D) Pupal ovaries mutant for ovo^{D1rS1}. (E) Pupal ovary from the agametic *ovo* allele combination, ovo^{D1rS1}/lzl^{G}. The unmarked arrow points to egg chamber. e, egg chambers with β-galactosidase expressing nurse cells; m, mature egg chambers; g, germ cells stained with β-galactosidase. Horizontal bar is a relative measure of magnification.

the *otu-lacZ* construct appears to be a more robust marker for germ cells than *BC69*.

A heat shock promoter-*otu* fusion construct can rescue *otu* mutations: To complement the descriptive analysis of the *otu* mutant phenotype, we undertook a series of experiments that identified the developmental stages when *otu* function was required. A fusion gene was constructed in which genomic DNA that included the *otu* coding region was placed under the control of the heat shock *hsp-70* promoter (*hs-otu*). We tested the ability of *hs-otu* to support oogenesis in the absence of a wild-type copy of the endogenous *otu* gene. Female flies mutant for different *otu* allele combinations and carrying the *hs-otu* construct were grown under the heat shock regimen (1 hr daily exposure to 37° followed by incubation at 25°) throughout development. Complete rescue of both fertility and fecundity were obtained with only the weakest *otu* allele, otu^{14} (Table 1). More severe alleles that cause agametic ($otu^{P\Delta 1}$ and otu^{10}) or tumorous (otu^{1}, otu^{3}, otu^{13}, $otu^{P\Delta 3}$) phenotypes showed substantial morphological suppression but were more variable in their fertility. Despite this variability, it is clear that in the presence of *hs-otu*, all *otu* mutant combinations showed an increase in the production of mature egg chambers. This indicates that *hs-otu* can partially compensate for the absence of endogenous *otu* product. Surprisingly, we found that *otu* mutant females had the same ovarian phenotype with one or two copies of the construct (Table 1). This suggests that the partial suppression does not result from insufficient *otu* activity. Instead, *hs-otu* may not be expressed in all the appropriate cells or developmental stages required for oogenesis, or a more continuous pattern of *otu* expression is required than that obtained by the daily heat shock routine.

Determining when *otu* function is required for oogenesis: Our strategy was to use *hs-otu* to induce *otu* activity at selected times in development in flies otherwise lacking *otu* function. In these experiments, flies were cultured for various times under two different temperature regimes. The heat shock regimen consisted of a daily 1-hr exposure to 37° followed by incubation at 25°. The nonheat-shock condition consisted of continuous culturing at 18°. To facilitate the analysis of the ovarian phenotypes, the experimental gonads were separated into four categories based on morphological criteria. Group 1 consisted of agametic ovaries noted by the absence of egg chambers. Group 2 consisted of ovaries containing only egg cysts of the ovarian tumor phenotype. Group 3 ovaries had one or more egg chambers in which the germ cells had limited oogenic differentiation. These chambers contained variable numbers of nurse-like cells that had large polytenized chromosomes, a phenotype common to certain hypomorphic *otu* alleles (KING and RILEY 1982). No yolk deposition was detected, indicating arrest before oogenic stage 7 (KING 1970). Group 4 ovaries contained one or more vitellogenic (post stage 7) egg cysts. These chambers had a visible oocyte with substantial yolk and chorion

TABLE 1

The effect of the *hs-otu* minigene on the *otu* mutant phenotype

Genotype[a]	Percentage fertile females[b]	Fecundity (progeny/female/week)	Ovary phenotype[c] without *hs-otu*	Ovary phenotype with *hs-otu*
$otu^{P\Delta 1}/otu^{P\Delta 1}$; *hs-otu*/+	0.0 (26)	—	Quiescent	Differentiated
$otu^{P\Delta 1}/otu^{P\Delta 1}$; *hs-otu/hs-otu*	0.0 (24)	—	Quiescent	Differentiated
$otu^{P\Delta 1}/otu^{10}$; *hs-otu*/+	0.0 (26)	—	Quiescent	Differentiated
otu^{10}/otu^{10}; *hs-otu*/+	9.7 (41)	<5	Quiescent	Differentiated
otu^{1}/otu^{1}; *hs-otu*/+	19.3 (31)	<5	Tumorous	Differentiated
otu^{13}/otu^{13}; *hs-otu*/+	5.7 (35)	<5	Tumorous	Differentiated
otu^{3}/otu^{3}; *hs-otu*/+	10.0 (48)	<5	Tumorous	Differentiated
$otu^{P\Delta 3}/otu^{P\Delta 3}$; *hs-otu*/+	40.0 (25)	32	Tumorous	Wild type
otu^{7}/otu^{7}; *hs-otu*/+	6.3 (48)	<5	Differentiated	Not done[d]
otu^{5}/otu^{5}; *hs-otu*/+	4.0 (24)	<5	Differentiated	Not done[d]
$otu^{P\Delta 2}/otu^{P\Delta 2}$; *hs-otu*/+	58.0 (12)	>50	Differentiated	Not done[d]
otu^{14}/otu^{14}; *hs-otu*/+	100.0 (50)	>50	Differentiated	Not done[d]

[a] Complete genotypes: otu^{10}, sn^{3} otu^{10} *v f*; otu^{14}, *y* otu^{14}; otu^{1}, sn^{3} otu^{1} *v f*.

[b] Number of females tested in parentheses.

[c] Quiescent ovaries lack egg cysts. They appear to be mostly empty of germ cells. Tumorous egg cyst contain hundreds of small undifferentiated germ cells. Differentiated eggs cysts are arrested at late oogenic stages. In these cysts the germ line has differentiated into nurse cells and oocytes and chorion deposition has occurred. The nurse cell nuclei are usually polytene.

[d] Not done because the unsuppressed phenotype is very similar to wild type.

deposition. Often the oocyte had completely displaced the nurse cells and dorsal appendages were present.

The *hs-otu* construct was tested in $otu^{P\Delta 1}/otu^{10}$ mutant females. The $otu^{P\Delta 1}$ allele is a deletion of the entire *otu* coding region (GEYER *et al.* 1993; SASS *et al.* 1993). The agametic ovarian phenotype of $otu^{P\Delta 1}/otu^{10}$ is identical to otu^{10} or $otu^{P\Delta 1}$ homozygotes. When $otu^{P\Delta 1}/otu^{10}$ females that did not carry *hs-otu* were cultured under the heat shock regimen throughout development (control, Figure 6), over 60% of the ovaries were agametic with the remainder containing small tumorous egg chambers. The addition of *hs-otu* resulted in a small increase in the proportion of gonads containing one or more egg chambers at 18°, indicative of residual expression from *hs-otu* under nonheat shock conditions. However, the majority of ovaries (63%) remained severely mutant (groups 1 or 2).

No additional phenotypic suppression was seen when *hs-otu* expression was limited to prepupal periods. In fact, there appeared to be a deleterious effect on oogenesis resulting from embryonic heat induction. In every case, heat shock treatment during embryogenesis resulted in a more severe average ovarian phenotype than if limited to later developmental stages. For example, heat shock induction from the embryonic to larval (E-L), embryonic to pupal (E-P), or embryonic to adult (E-A) stages caused a more severe phenotype than when genotypically identical flies were heat shocked only at postembryonic stages (compare with L, P, or A, respectively, in Figure 6). These observations could reflect either a negative effect of high temperature or *otu* expression on embryonic germline development.

A modest phenotypic suppression occurred when *otu* induction was limited to the pupal stage (P, Figure 6). There was a reduction in the frequency of agametic ovaries and a minor increase in the ovaries containing the most mature chambers. Nevertheless, the majority of ovaries were still of groups 1 or 2. Only when *otu* was induced during the adult stage was there substantial phenotypic suppression. Heat shock induction limited to the adult stage (for 7 days) was sufficient to eliminate agametic ovaries and allow increased production of oogenic egg chambers (A, Figure 6), while the highest frequency of mature egg chambers occured when the heat shock regimen included both pupal and adult stages (P-A, Figure 6). These data indicate that *otu* function is required for oogenesis during pupal and particularly adult stages.

The OTU 98-kD isoform allows the proliferation of *XX* germ cells: The *otu* gene produces two *otu* isoforms of 98 and 104 kD by the alternative RNA splicing of a single exon (STEINHAUER and KALFAYAN 1992). These two isoforms appear to have different roles in oogenesis. The 104-kD isoform is required for the differentiation of nurse cells and oocytes, as mediated by the *Sxl* gene (BAE *et al.* 1994). The 98-kD product is needed for germ cell proliferation in a manner dependent on the sexual identity of the somatic gonad (NAGOSHI *et al.* 1995). To further examine the functon of the 98-kD product in oogenesis, we created a construct in which a cDNA specific for the 98-kD isoform was fused to the heat shock *hsp-70* promoter (*hs-98*) (NAGOSHI *et al.* 1995). This construct allows induction of only the 98-kD *otu* product independent of the normal regulators of *otu* transcription and RNA splicing.

Flies mutant for the most severe *otu* alleles (otu^{10}/otu^{10} or $otu^{10}/otu^{P\Delta 1}$) and carrying *hs-98* were heat treated throughout development and their ovarian morphol-

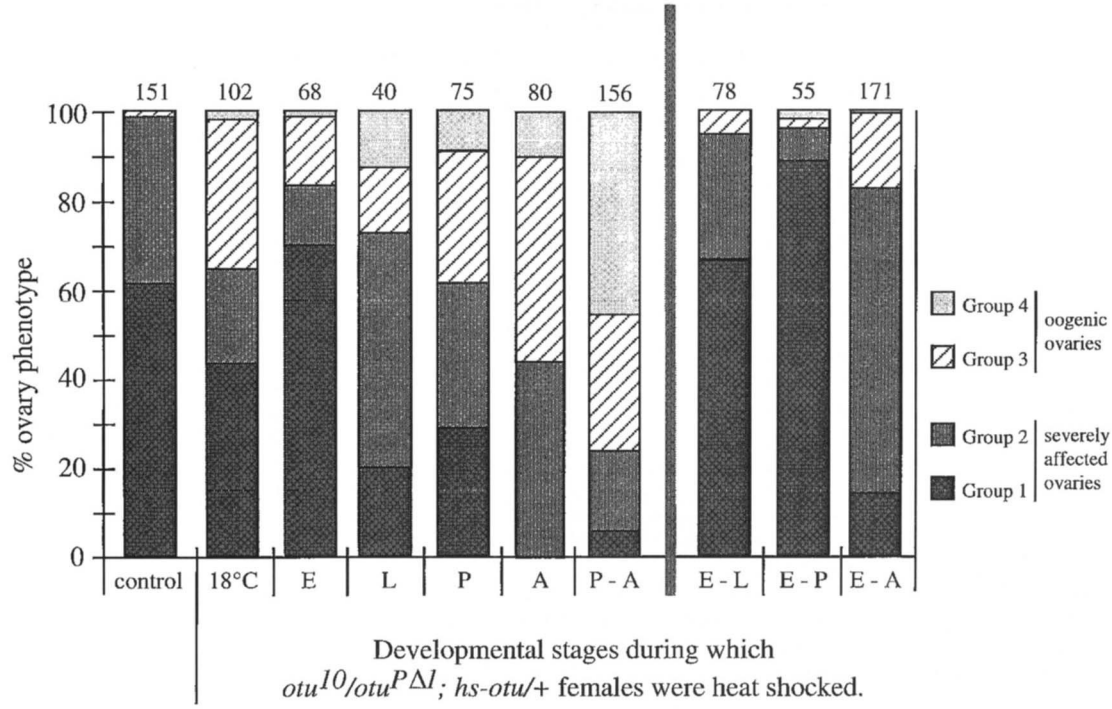

FIGURE 6.—The effect of *otu* induction on oogenesis at various developmental stages. $otu^{10}/otu^{P\Delta 1}$ flies carrying the *hs-otu* construct were either grown at 18° or cultured under our heat shock regimen (daily 1 hr exposure to 37° followed by incubation at 25°). Heat shock control, $otu^{10}/otu^{P\Delta 1}$ flies without *hs-otu* cultured under the heat shock regimen throughout development. The following categories represent experimental trials where genotypically identical $otu^{10}/otu^{P\Delta 1}$ flies carrying one copy of the *hs-otu* construct were grown under different culture conditions. 18°C, flies grown continually at 18°; E, heat shock regimen limited to embryogenesis (24 hr post egg lay); E-L, heat shock regimen during the embryonic and larval periods (from egg lay until wandering larvae); L, heat shock regimen only during larval stages; E-P, heat shock regimen during the embryonic, larval and pupal stages; L-A, heat shock regimen during the larval, pupal and adult stages; P, heat shock regimen during the pupal stage only; P-A, heat shock regimen during pupal and adult stages; A, heat shock regimen for 7 days during adulthood. Flies cultured as adults under the heat shock regimen were aged for 3–5 days before dissection (except for A, which were dissected after 7 days). Flies cultured as adults at 18° were aged for 7–10 days before dissection. Mutant phenotypes: group 1, agametic gonads; group 2, tumorous egg chambers; group 3, nurse cells but no yolk deposition; group 4, ovaries with mature egg chambers. Numbers on top of the vertical bars indicate the number of gonads tested.

ogy examined. In the absence of *hs-98,* primarily agametic ovaries were observed (Figure 7A). In contrast, flies carrying the *hs-98* construct had relatively large ovaries containing many tumorous egg chambers (Figure 7B). After prolonged heat treatment, occasional cysts were found with nurse-like cells that had an extreme polytene phenotype (Figure 7C). These results demonstrate that the induced expression of the OTU 98-kD function is sufficient to allow *XX* germ cell proliferation and the formation of egg chambers but cannot consistently support the differentiation of the germ cells into later oogenic stages.

DISCUSSION

***otu* is required in the pupal and adult ovary:** The most severe *otu* alleles produce agametic adult ovaries that are devoid of egg chambers and have variable numbers of germ cells. The same alleles have no effect on spermatogenesis. We investigated whether this sex-specific phenotype could be seen during the embryonic and larval stages when sexual dimorphism of the germline first becomes apparent. The contention that *otu* has a role in the determination of germline sexual identity would be supported by any demonstration that *otu* mutations affect early stages of female germline development. However, we found no evidence that even the most severe *otu* alleles affected the development of *XX* germ cells during the embryonic or larval stages. The earliest detectable aberration in ovarian morphology occurred during pupariation and continued into the adult stage.

These observations are consistent with temperature-shift studies using a heat-inducible *otu* construct. We found that *otu* activity induced during the pupal and particularly the adult stages suppressed the *otu* mutant phenotype. In contrast, *otu* expression limited to embryonic or larval stages failed to rescue *otu* mutants and, in fact, may have had a deleterious effect on oogenesis. Based on these results, we do not believe that *otu* is required for the initiation of germline sex determination. The postlarval requirement for *otu* is more consistent with this gene responding to a preexisting sex determination signal, perhaps coming from the soma (NAGOSHI *et al.* 1995).

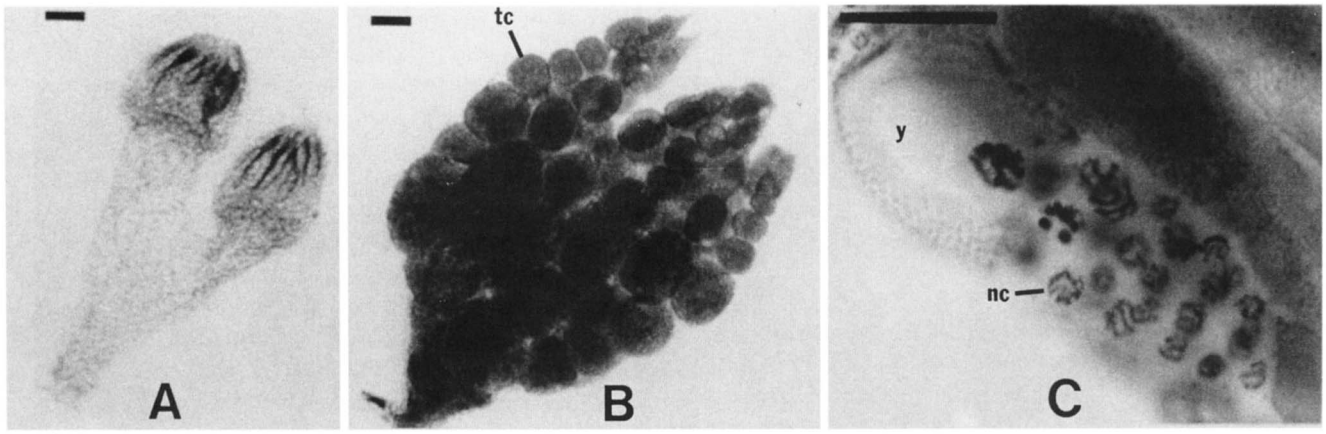

FIGURE 7.—Rescue of the *otu* mutant phenotype by induced 98-kD activity. (A) Two lobes from an otu^{10}/otu^{10} ovary showing the agametic phenotype. Ovarioles are present but are devoid of egg chambers. (B) Ovary from otu^{10}/otu^{10} female carrying one copy of the *hs-98* construct. The flies were cultured under the heat shock regimen during pupariation and for 5 days after eclosion. The ovaries contain many tumorous (tc) egg chambers. (C) Egg chamber from otu^{10}/otu^{10} female carrying one copy of *hs-98*. Prolonged heat-induction of *hs-98* activity can occasionally produce a few chambers with polytene nurse-like cells (nc) and yolk deposition (y). Female was cultured under the heat shock regimen during pupariation and for 10 days after eclosion. Horizontal bar is a relative measure of magnification.

Is *ovo* required for germ cell viability in the embryo? The strongest evidence supporting the involvement of *ovo* in early germline sex determination comes from the report that severe *ovo* mutations cause the loss of *XX* pole cells during early gastrulation (OLIVER *et al.* 1987, 1990; PAULI and MAHOWALD 1990). It was found that in a cross between $ovo^{D1rS1}/+$ and ovo^{D1rS1}/Y a portion of the resulting embryos had reduced numbers of germ cells by gastrulation. Presumably, the affected embryos were ovo^{D1rS1} homozygotes, although the experiments were not designed to allow identification of either embryonic sex or genotype. Our experiments using the same *ovo* allele found no evidence of reduced germ cell number in ovo^- animals during middle gastrulation (embryonic stages 7–11). In these studies, we purposely examined later embryonic stages than those in which ovo^- germ cells were first reported to be lost. This was to make certain that we did not miss the critical period of *ovo* function and was based on the assumption that if the adult agametic phenotype resulted from these embryonic aberrations, then the reduction in germ cell number should be maintained, if not exacerbated, throughout development.

The discrepancy between our study and previous reports is puzzling. Although the same *ovo* allele was tested, it is possible that potential differences in the genetic background and culture conditions used in the different studies affected the severity of the *ovo* phenotype. In fact, variability in the effect of *ovo* mutations on embryonic pole cell numbers had been previously reported and attributed to background effects (OLIVER *et al.* 1987, 1990). We attempted to control for such variations in two ways. First, the environmental and genetic context was held constant by comparing sibling embryos grown in the same vials. Second, different ovo^{D1rS1} strains were tested in which portions of the *X* chromosome and the autosomes had been replaced by genetic techniques (data not shown). These experiments gave results indistinguishable from the data in Figure 2.

It is possible that the previously observed loss of ovo^- pole cells during the embryonic period is a variable and infrequent consequence of the absence of zygotic *ovo* activity. This could occur if a maternal contribution of *ovo* function can usually, but not always, support early germline development in the absence of zygotic *ovo*. Supporting this possibility is the finding that maternally contributed *ovo*-specific RNAs are localized to pole cells and present up to embryonic stage 8 (MÈVEL-NINIO *et al.* 1995). An alternative explanation is that *ovo* mutations reduce germ cell numbers during early gastrulation, but that this deficit is compensated for by the time the pole cells invaginate. In either case, it seems unlikely that the inconsistent or temporary reduction in ovo^- embryonic germ cell numbers can account for the consistently severe adult mutant phenotype.

Our data demonstrate that in *otu* and *ovo* mutants, *XX* germ cells are generally and most consistently affected during the period when stem cells are proliferating and differentiating into cystoblasts. In fact, germline stem cells accumulate as late as the adult stage in severe otu^- and ovo^- females, although these cells appear to be aberrant in their distribution among the ovarioles and in their ability to express germline-specific markers. While the frequent formation of empty adult ovarioles caused by these mutations might result from cell death, it could also be attributed to defects in germ cell proliferation or in the segregation of germ cells among the ovarioles. For example, mutant germ cells might be arrested at a stage when they are unable to correctly interact with the developing ovarioles. This could result in a haphazard distribution of oogonial cells in the

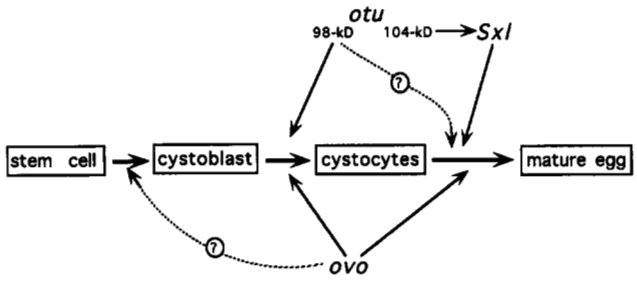

FIGURE 8.—Model for *otu, ovo, bam,* and *Sxl* functions in oogenesis. A diagram of early stages in oogenesis is shown with the proposed times when *otu, ovo, bam,* and *Sxl* functions are required. The *ovo* gene is required at several stages in oogenesis and appears to act independently of *otu* (NAGOSHI *et al.* 1994). The *bam* gene may be required for early cystoblast divisions (LIN *et al.* 1994) and appears to overlap in function with the OTU 98-kD isoform. The OTU 104-kD product is required for later stages in oogenesis and acts through the *Sxl* gene. Dashed arrows with question marks indicate possible additional functions.

ovarioles with the loss of unincorporated cells. Therefore, our observations are most easily explained if *ovo* mutations cause defects in germ cell proliferation and differentiation.

The functions of the OTU isoforms: Previous studies suggested that the two OTU isoforms had discrete and specific functions during oogenesis. We proposed that the OTU 98-kD isoform is required for the proliferation of germ cells in the ovary. This was based on two observations: the 98-kD product is required for the accumulation of *XY* germ cells when developing in a female somatic gonad (NAGOSHI *et al.* 1995) and alleles that specifically inactivate the 104-kD isoform, but allow 98-kD isoform expression, produce tumorous egg chambers (STEINHAUER and KALFAYAN 1992; BAE *et al.* 1994). Further support for this hypothesis comes from our finding that the induced expression of the 98-kD product can cause agametic ovaries to produce tumorous egg chambers. This demonstrates that the OTU 98-kD isoform can support *XX* germ cell proliferation during oogenesis. Subsequent development requires the activity of the OTU 104-kD isoform and the *Sxl* gene. This was demonstrated by epistasis experiments in which the constitutive expression of the *Sxl* gene completely rescued *otu* mutations that disrupted OTU 104-kD function but allowed expression of the 98-kD product (BAE *et al.* 1994). In contrast, in the absence of both isoforms no suppression by constitutive *Sxl* activity occurs (PAULI *et al.* 1993; BAE *et al.* 1994).

Taken together, these observations lead to a model in which the proliferation of the *XX* germline is controlled by the OTU 98-kD function, while differentiation of the germ cells depends on the 104-kD isoform acting through the *Sxl* gene (Figure 8). However, an alternative view of *otu* action has recently been proposed. Using isoform-specific constructs, it was shown that the 104-kD product can provide all necessary *otu* functions for oogenesis (SASS *et al.* 1995). In contrast, the 98-kD isoform appeared to be nonessential with only a minor function during late oogenic stages. These data suggest that the multiplicity of phenotypes associated with *otu* derive primarily from the activity of a single product. At this point, we cannot reconcile these divergent observations and conclusions.

In summary, the first consistent morphological defect observed for the most severe *ovo* and *otu* mutations occurs in the pupal ovary. Our data do not preclude the possibility that mutations in either of these genes have earlier effects. However, we demonstrate that in *ovo* and *otu* mutants, the majority of *XX* germ cells survive and express some appropriate germline-specific genes as late as pupal and adult stages of development. We also provide evidence that the alternatively spliced OTU 98-kD isoform can support female germ cell proliferation, if induced in *otu* mutant ovaries. This suggest a role for this product in early stages of oogenesis.

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