A specific ovarian tumor protein isoform is required for efficient differentiation of germ cells in Drosophila oogenesis

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Mutations within the ovarian tumor (otu) gene result in abnormal ovarian development. It has been proposed that otu phenotypes result from abnormal germ cell division and differentiation. To understand better what role otu performs in oogenesis we have undertaken an analysis of protein expression from the otu locus. Anti-otu antibodies recognize two proteins from Drosophila ovary extracts with apparent molecular masses of 98 and 104 kD. Sequence analysis of otu cDNAs suggests that these proteins are translated from two mRNAs generated by alternative splicing of a 126-bp exon between the sixth and seventh exon of the smaller transcript. Analysis of otu protein expression in eight mutants indicates a correlation between the accumulation of the 104-kD isoform and predifferentiated germ cells and suggests that there is a developmental shift in the accumulation of the two isoforms upon differentiation of germ cells. Furthermore, the 104-kD isoform appears to be required for efficient differentiation of germ cells. Immunostaining of otu proteins is restricted to the cytoplasm of germ cells, and a rapid loss of oocyte immunostaining during stage 11 suggests that there is a rapid and selective degradation of otu proteins within the oocyte but not within its 15 interconnected nurse cells.

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pathologies. Seventeen ethylmethane sulfonate (EMS)-induced, and 13 P-element-induced, or P-element-derived, otu mutants have been recovered (Gans et al. 1975; Mohler 1977; Digan 1980; King and Riley 1982; King et al. 1986; Mulligan et al. 1988; G. Sass and D. Mohler, unpubl.). The phenotypes displayed by otu mutants are divided into three developmental classes that highlight specific steps within oogenesis which may be affected by or under the control of the otu gene (King et al. 1986). An otu mutant is classified by the predominant phenotype displayed within the ovary; however, multiple phenotypes may be present in any given ovary.

The quiescent (QUI) class is the most severe and represents mutants in which germ cells fail to proliferate, causing ovarioles to appear as empty sheaths of somatic tissue (King and Riley 1982). Mutants from the oncogenic (ONC) class have lost the ability to control cystocyte divisions: the usual four incomplete rounds of mitosis are replaced by complete and continued cell divisions, giving rise to tumorous egg chambers filled with hundreds of undifferentiated cystocytes (King 1979). The differentiated (DIF) class encompasses mutants with ovaries that predominantly contain egg chambers exhibiting some form of differentiation. Cystocyte divisions are not always precise, and some alleles exhibit odd numbers of nurse cells or no oocyte. Those egg chambers that contain oocytes usually proceed through oogenesis to what has been called a "pseudo-12" (p12) stage but do not complete development (King et al. 1986, Storto and King 1988).

Initial molecular characterization of the otu gene and its products identified a predominant 3.2-kb ovary-specific transcript (Mulligan et al. 1988). Sequence analysis of the otu gene suggested that otu produced a single 811-amino-acid protein with a 12% proline content. Here, we describe a second otu protein isoform expressed in ovaries that is generated through alternative splicing and examine alterations in expression of the two isoforms in eight otu mutants. Our results suggest that a specific isoform is required for efficient differentiation of the germ cells and that differentiation may bring about a shift in the expression of the two protein isoforms. Immunolocalization of otu proteins in situ suggests that otu is a germ cell-specific cytoplasmic protein and that its expression is differentially regulated between nurse cells and oocytes through a rapid and selective degradation of otu protein within the oocyte at stage 11.

Results

Two protein isoforms are evident from analysis of otu gene products

Previous sequence analysis of the otu gene and several otu cDNAs suggested that otu produced a single protein of 811 amino acids (Steinhauer et al. 1989). We raised polyclonal antibodies that recognize two specific regions of this otu gene product: amino acids 253-671 and 670-811. Coding regions used in lacZ-otu fusion constructs are shown below a schematic representation of an otu cDNA (Fig. 1A). The unmodified expression vector (pWR590) produces a truncated form of β-galactosidase with a molecular mass of ~80 kD (Fig. 2, lane 2), whereas fusion proteins produced from constructs 1 and 2 have masses of ~140 and 90 kD, respectively (Fig. 2, lanes 3, 4).

Rabbit polyclonal antibodies against the two domains of otu protein were purified on fusion protein-affinity columns to generate anti-otu(253-671)- and anti-otu(670-811)-specific antisera. Antisera specificities were examined in Escherichia coli extracts and in crude

Figure 1. The structures of otu cDNAs and fusion proteins. [A] The otu gene structure with translation initiation and termination sites indicated is shown above the schematic exon structure of cDNA 3-2. Approximate locations of lesions from three P-element-induced or -derived mutations are shown at the 5' end of the otu gene. The coding sequences of the otu gene ligated into the pWR590 expression vector are identified in constructs 1 and 2. [B] Schematic exon structure of cDNA 4-1. Differences between cDNA 4-1 and cDNA 3-2 include the lack of 68 nucleotides from cDNA 3-2 at the 5' end of cDNA 4-1, the truncation of cDNA 4-1 at an internal EcoRI site of cDNA 3-2, and the addition of an alternate exon (6a) in cDNA 4-1 between exons 6 and 7 of cDNA 3-2.
Figure 2. Analysis of the specificity of two anti-otu antisera. [Lanes 1-4] Total protein staining of E. coli extracts after fractionation by SDS-PAGE and transfer to nitrocellulose. The positions of the proteins expressed from the original expression vector [lane 2, 80 kD], and the two fusion constructs: lacZ-otu(253-671) [lane 3, 140 kD] and lacZ-otu(670-811) [lane 4, 90 kD] are indicated by arrowheads. [Lane 1] An extract of the parent E. coli strain [MV1184] without an expression vector, [lanes 5-7, 8-10] Equivalent blots of extracts from E. coli expressing lacZ-otu(253-671) [lanes 5, 8], lacZ-otu(670-811) [lanes 6, 9], or 10 μg of crude ovary extract from Canton-S flies [lanes 7, 10]. Lanes 5-7 and 8-10 were incubated with anti-otu(253-671) and anti-otu(670-811) antisera, respectively. The immunostaining proteins below the intact fusion proteins [lanes 5, 9] appear to be degradation products of the fusion proteins. Two proteins from Canton-S ovary extracts [at 104 and 98 kD] immunostain with both antisera. The light immunostain at ~45 kD (lane 10) appears to be nonspecific staining of the parental E. coli strain (MV1184) without an expression vector; the 90-kD fusion protein and does not cross-react with the 98-kD protein and a protein expressed from the original expression vector [lane 2, 80 kD], and the two fusion constructs: lacZ-otu(253-671) [lane 3, 140 kD] and lacZ-otu(670-811) [lane 4, 90 kD] are indicated by arrowheads. [Lane 1] An extract of the parent E. coli strain [MV1184] without an expression vector, [lanes 5-7, 8-10] Equivalent blots of extracts from E. coli expressing lacZ-otu(253-671) [lanes 5, 8], lacZ-otu(670-811) [lanes 6, 9], or 10 μg of crude ovary extract from Canton-S flies [lanes 7, 10]. Lanes 5-7 and 8-10 were incubated with anti-otu(253-671) and anti-otu(670-811) antisera, respectively. The immunostaining proteins below the intact fusion proteins [lanes 5, 9] appear to be degradation products of the fusion proteins. Two proteins from Canton-S ovary extracts [at 104 and 98 kD] immunostain with both antisera. The light immunostain at ~45 kD (lane 10) appears to be nonspecific staining of the highly abundant vitellogenin proteins from the ovary extracts. The positions of molecular mass markers are shown at left.

Several otu mutants exhibit alterations in the expression and/or size of otu proteins

We wished to know whether any of the ovarian pathologies of otu mutants could be correlated with specific alterations in expression of the two otu isoforms. The expression of otu proteins in selected alleles was examined by Western blot [see below]. This analysis led us to sequence specific genomic regions of four otu mutants. The two ONC mutants otu11 and otu13 were sequenced across the alternate exon and because these mutations were induced in the same parental chromosome, sequence differences between these two alleles should identify mutations rather than background polymorphisms. A 408-bp genomic fragment of otu11 and otu13 that includes the alternate exon [Fig. 3, nucleotides 2643-3050] was amplified by polymerase chain reaction [PCR], and the amplified DNA was sequenced. Genomic sequence of otu13 indicated a G → A base substitution at nucleotide 2779 [Fig. 3]. This transition alters the 3'-splice acceptor site adjacent to the 5' end of the alternate exon by converting the invariant AG dinucleotide, required for splice acceptor site function, to AA. The genomic sequence of otu11 contained a G → A base substitution at nucleotide 2793, which converts the cysteine at...
amino acid 5A to tyrosine (Fig. 3). A third ONC mutant (otu^A3) is known to have a deletion of -450 bp upstream of the cDNA start site but has an unaltered coding region [G. Sass et al., in prep., Fig. 1A].

Of the four ONC mutants examined by Western blot, three (otu^11, otu^13; Fig. 4, lanes 2, 4, 5) accumulate higher proportions of the large protein, although the absolute amount of both isoforms is reduced dramatically in two of these mutants [Fig. 4, lanes 4, 5]. In the case of otu^A3 the 98-kD isofrom is either not expressed or is expressed below the level of detectability. In contrast, the ONC allele otu^13 [Fig. 4, lane 3] displays nearly wild-type levels of protein but does not accumulate detectable levels of the 104-kD isofrom. The absence of the larger isofrom is expected because of the splicing site mutation at the alternate exon of otu^13. These data suggest that ONC mutants are correlated with reduced amounts of the 98-kD isofrom with respect to the normal abundance of this isofrom in wild-type ovaries.

As with otu^11 and otu^13 two DIF alleles otu^5 and otu^14 were sequenced within specific regions of the otu gene. Because both otu^5 and otu^14 accumulate smaller proteins, it seems likely that these alleles contained nonsense mutations. On the basis of sizes of the truncated proteins in otu^5 and otu^14 extracts, an estimate was made of the region within the otu gene where premature translation termination signals might be located. Fragments with 219 bp of genomic DNA (nucleotides 3947–4166; Steinhauser et al. 1989) from otu^5 and otu^14 were PCR amplified, cloned, and sequenced. Sequences of both mutants contained C→T base substitutions that created stop codons in exon 7, after residue 621 of otu^A13 and residue 655 of otu^14 [numbered with respect to the 98-kD isofrom].

Antiserum that recognizes the carboxy-terminal region of otu proteins does not recognize otu proteins in

Figure 4. Western analysis of otu mutants. Eight mutants were analyzed, four ONC alleles [otu^11 (lane 2), otu^13 (lane 3), otu^14 (lane 4), otu^A3 (lane 5)], and four DIF alleles [otu^5 (lanes 6 and 11), otu^14 (lanes 7 and 12), otu^A4 (lane 8), otu^A5 (lane 9)]. A 20-μg aliquot of ovary extract was loaded for each mutant, except for otu^5 where 40 μg was loaded, and 10 μg of wild-type (Canton-S) ovary extract was loaded in lanes 1 and 10. Lanes 1–9 were incubated with anti-otu(670–811) antiserum, lanes 10–12 were incubated with anti-otu(253–671) antiserum. The two otu protein isoforms identified in Fig. 2 are indicated [104 and 98 kD], and truncated forms of these proteins from the otu^5 and otu^14 alleles are identified by the asterisk (*). Note that these truncated forms are not recognized by the anti-otu(670–811) antiserum [lanes 6, 7]. The positions of molecular mass markers are shown at left.
extracts derived from *otu* \(^5\) and *otu* \(^{14}\) (Fig. 4, lanes 6, 7). However, in extracts from both mutants, antisera that recognizes the central portion of the *otu* proteins (amino acids 253–671) immunostains two proteins with lower molecular masses than the native *otu* proteins. In *otu* \(^5\) extracts, 82- and 76-kD polypeptides were detected (Fig. 4, lane 11), whereas in *otu* \(^{14}\) extracts, 86- and 81-kD polypeptides were observed (Fig. 4, lane 12). Calculated molecular masses for the two isoforms are 77.2 and 72.3 kD for *otu* \(^5\), and 80.6 and 75.8 kD for *otu* \(^{14}\). These calculated sizes are consistent with the respective masses observed, again with the assumption that proline content affects mobility. Two other DIF mutants, *otu* \(^{14}\) and *otu* \(^{23}\) (Fig. 4, lanes 8, 9), exhibit the same sized *otu* polypeptides as wild-type flies but at considerably reduced levels. In contrast to the ONC mutants, DIF mutants appear to maintain a more nearly wild-type ratio of the two isoforms with respect to each other.

The truncated proteins of *otu* \(^5\) and *otu* \(^{14}\) are capable of promoting the early stages of oogenesis, suggesting that 190 amino acids of the carboxyl terminus are not required for proper cyst formation or differentiation. Inability of these mutants to complete oogenesis may be the result of a loss of function associated with the carboxy-terminal domains but could also result from low protein levels as seen in the Western blot, a result of reduced stability of the truncated proteins. Table 1 summarizes the phenotypes and molecular lesions associated with the *otu* alleles examined.

**Fertile heteroallelic combinations alter *otu* protein expression**

Although most heteroallelic combinations of *otu* yield intermediate phenotypes, the two ONC alleles that express relatively high levels of *otu* protein (*otu* \(^{11}\) and *otu* \(^{13}\)) complement several DIF alleles to fertility. Most of these transheterozygotes have ovaries that contain substantial numbers of tumorous chambers or chambers lacking oocytes, but the chambers that form properly complete development and produce fertile eggs. The exception is the *otu* \(^{11}\)/*otu* \(^{14}\) heterozygotes, which have nearly normal ovarian morphology (Storto and King 1987). Using the DIF alleles *otu* \(^5\) and *otu* \(^{14}\) and the anti-*otu*(670–811) antisera that does not recognize the truncated proteins from these alleles, we examined the effects of these DIF alleles on the accumulation of *otu* proteins expressed from *otu* \(^{11}\) and *otu* \(^{13}\) alleles in transheterozygotes.

**Localization of *otu* protein in Canton-S and mutant alleles**

We examined the temporal and spatial distribution of *otu* proteins in wild-type and *otu* mutant ovary tissue sections by immunocytochemistry. Immunostaining was evident in cytoplasm of cystocytes, nurse cells, and oocytes, but no *otu* protein was detected in nuclei or follicle cells. Intense immunostaining is present in germarial stages.

**Table 1. Selected alleles of the *otu* locus and their associated lesions**

| Allele | Class | Ovarian phenotypes | Molecular lesion |
|--------|-------|-------------------|-----------------|
| *otu* \(^{11}\) | ONC | homzygotes | G → A substitution at position 2793, Cys → Tyr transition at position 5A of alternate exon |
| *otu* \(^{13}\) | ONC | homzygotes | G → A substitution at position 2779; alters the splice acceptor at the 5' end of exon 6a |
| *otu* \(^5\) | ONC | quiescent | unknown |
| *otu* \(^{23}\) | ONC | quiescent | ~450-bp deletion 5' of cDNA 3-2 start site [Fig. 1A] |
| *otu* \(^{14}\) | DIF | differentiated | C → T substitution at position 4018; 190-amino-acid truncation after residue 621 |
| *otu* \(^{29}\) | DIF | differentiated | C → T substitution at position 4120, 156-amino-acid truncation after residue 655 |
| *otu* \(^{32}\) | DIF | differentiated | ~500-bp insertion near cDNA 3-2 start site [Fig. 1A] |

Position numbers are as in Fig. 3 and Steinhauer et al. [1989]. Residue numbers for the truncated proteins are with respect to the 98-kD isofrom. (ND) Not determined.
Figure 5. Western analysis of heteroallelic combinations of otu mutant alleles. Heteroallelic combinations were made of two ONC mutants \(\text{otu}^{11}\) and \(\text{otu}^{13}\) with each of two DIF mutants \(\text{otu}^{12}\) and \(\text{otu}^{14}\). The genotypes of the flies from which the ovary extracts were made are indicated above each lane. Only the wild-type Canton-S (CS), \(\text{otu}^{11}\), and \(\text{otu}^{13}\) alleles express otu proteins, which can be recognized by the antisera used to probe this Western blot. Lane 1 has 10 μg of ovary extract loaded; the other lanes have either 20 μg (lanes 2, 3, 5, 7, 8, 10, 12) or 40 μg (lanes 4, 6, 9, 11) loaded. The 40 μg loadings compensate for heterozygotes with only one allele that expresses recognizable protein.

Discussion

Alternative splicing of otu pre-mRNA generates two protein isoforms upon translation

We have described a cDNA from the otu locus that contains an extra exon between the sixth and seventh exons of the transcript reported previously [Steinhauer et al. 1989]. Antibodies raised against fusion proteins generated with two different regions of otu-coding sequences each recognize two protein isoforms of sizes that correspond to the molecular masses predicted by the conceptual translation of the two cDNAs. Western analysis showed that both isoforms were altered in otu mutants, either in mobility or relative abundance. Particularly convincing evidence for the relevance of both bands is the absence of the larger isoform in \(\text{otu}^{13}\) and truncation of both isoforms in \(\text{otu}^{12}\) and \(\text{otu}^{14}\). Each of these alterations has been correlated with a point mutation that disrupts a splice junction of the alternate exon \(\text{otu}^{13}\) or creates a nonsense mutation \(\text{otu}^{12}\) and \(\text{otu}^{14}\). The splice site mutation in \(\text{otu}^{13}\) prevents appropriate splicing of the alternate exon that results in the absence of the larger isoform in \(\text{otu}^{13}\) ovary extracts. Although these three alleles have not been sequenced across the entire otu-coding region, the correlation of a specific point mutation with a predicted observable change in protein expression on Western blots is strong evidence that the identified lesions are responsible for the mutant phenotypes of these alleles. We conclude that two protein isoforms are derived from otu by an alternative splicing mechanism.

A shift in protein expression may coincide with cystocyte differentiation

Our results show a shift in accumulation of one otu protein isoform over the other that may coincide with differentiation of the germ cells. If a correlation exists between the state of differentiation and the expression of a specific otu isoform, then the ratio of the two isoforms in an extract will be a function of the degree of differentiation of germ cells within the ovary. Therefore, amounts of a specific isoform in extracts from ovaries with varying degrees of differentiation cannot be directly compared. For instance, ovary extracts from DIF mutants [mostly differentiated germ cells], which may be expressing greater amounts of the 104-kD isoform in predifferentiated germ cells than ONC mutants [mostly undifferentiated germ cells], may still accumulate less of this isoform because of the greater percentage of predifferentiated germ cells in ONC mutants. Therefore, only the total level of expression of both isoforms and the relative ratio of the two isoforms can be compared between phenotypes. Furthermore, the ratio of the isoforms in a specific mutant will not be constant because of variability in the degree of differentiation in different extracts. For instance, the two different extracts of \(\text{otu}^{11}\) ovaries [Fig. 4, lane 2; Fig. 5, lane 2] show varying ratios of the two isoforms, which presumably reflects varying degrees of differentiation in these ovary preparations. We have shown that ovary extracts from otu mutants, characterized by greater percentages of undifferentiated germ cells within their ovaries than wild-type flies, exhibit correspondingly greater ratios of the 104-kD isoform to the 98-kD isoform, whereas otu alleles characterized by more normal percentages of differentiated germ cells within their ovaries showed more normal ratios of the two isoforms. Moreover, when we experimentally alter
Figure 6. In situ localization of otu proteins in wild-type (Canton-S) and otu mutant ovaries. Frozen sections (6 μm) were incubated with anti-otu(670–811) antiserum (A,B,D,E), control antiserum (C), or anti-otu(253–671) antiserum (F). Localization of otu proteins was accomplished by the avidin–biotin peroxidase complex technique. (dc) Differentiated egg chamber. (fc) Follicle cells. (g) Germarium. (nn) Nurse cell nuclei. (o) Oocyte. (p12) Pseudo-12-stage egg chamber. (tc) Tumorous chamber. Numbers within egg chambers represent the developmental stage of the egg chamber. (A–C) Sections from wild-type (Canton-S) ovaries exhibit germarial staining and strong cytoplasmic staining in nurse cells and oocytes. Oocyte staining has disappeared by stage 11 (B). (C) Most of the immunostaining is removed by passing anti-otu(670–811) antiserum over a lacZ-otu(670–811) affinity column prior to incubation with the sections. (D) Sections from otu11 ovaries exhibit moderate staining in germaria and tumorous chambers and strong staining in differentiated egg chambers. (E) Immunostaining in otu13 ovaries is similar to that of otu11 ovaries. (F) Sections from otu14 ovaries exhibited light germarial staining, but no significant accumulation of otu proteins is seen in later stages of oogenesis as is seen in wild-type ovaries.
the degree of differentiation of the ovaries in which an otu allele is present through heteroallelic combinations, a shift in the ratio of the two isoforms results (i.e., the pattern of expression from the otu allele is shifted from a predominance of the 104-kD isoform in homozygotes to a predominance of the 98-kD isoform in otu/otu heterozygotes). Although it appears likely, we cannot conclude that normal undifferentiated germ cells express only the 104-kD isoform, because ovaries of most ONC alleles contain both differentiated and undifferentiated chambers. However, the ONC allele otu, which has unaltered coding sequences [Fig. 1 and Table 1] and contains only undifferentiated germ cells [G. Sass, unpubl.], does not accumulate detectable levels of the 98-kD isoform. The accumulated data suggest that differentiation of cystocytes into nurse cells and an oocyte alters the splicing pathway of otu pre-mRNA, shunting splicing toward removal of the alternate exon. This is not to say that expression of the 98-kD isoform brings about differentiation of cystocytes but is a result of that differentiation. Alternatively, differentiation could affect the stability of one mRNA or protein species more than the other.

A model for otu expression

It has been proposed that the otu gene product is required in increasing amounts throughout oogenesis and that different classes of otu mutants reflect phenocritical thresholds of functional otu protein expression [King and Riley 1982, King et al. 1986]. The fact that heteroallelic combinations of some ONC alleles with some DIF alleles yield fertile flies rather than flies with intermediate ovarian pathologies suggests that a second otu product exists and that each complementing allele provides one of the required functions [Storto and King 1987]. It is remarkable that the hypothesis developed by King and his colleagues from genetic and morphological analyses should, for the most part, predict observed molecular data so closely.

The three developmental classes (QUI, ONC, and DIF) that categorize otu phenotypes highlight three stages of oogenesis where otu gene products appear to have a major function: stem cell proliferation, control of germ cell divisions and differentiation, and oocyte development. The first two stages require only low levels of otu expression as evidenced by the reduced levels of otu proteins in two of the ONC mutants and all of the DIF mutants examined. Furthermore, the threshold of expression that separates the ONC and DIF phenotypes appears to be very narrow because among mutants that have unaltered coding regions, DIF mutants accumulate only slightly greater amounts of otu products than ONC mutants. Several observations lead us to conclude that later stages of oogenesis require greater accumulation of otu protein for normal oocyte development. First, results from an in situ analysis suggest that otu proteins accumulate to high levels in nurse cells of stage 11 egg chambers. Second, all DIF mutants examined accumulate much less protein than wild-type flies. The molecular lesions in otu and otu result in truncated otu proteins, and by immunocytochemical analysis [otu], the truncations appear primarily to destabilize the proteins and prevent their accumulation in later stages of oogenesis. Finally, heteroallelic crosses of otu with ONC alleles are fertile when the ONC alleles express high levels of otu proteins [otu and otu], but are sterile when the ONC alleles express low levels of otu proteins [otu and otu]. Thus, the termination of oogenesis around stage 12 in otu mutants can be overcome by providing a stable and abundant source of the 98-kD isoform.

We have suggested that a shift in expression coincides with differentiation of germ cells, but is there a requirement for a specific isoform at any specific stage of oogenesis? The control of cyst formation and differentiation appears to be sensitive to which otu isoform is expressed, as suggested by the observations outlined here. [1] Two ONC mutants otu and otu accumulate high levels of otu protein and yet give rise to tumorous ovaries, a more severe ovarian pathology than that of the DIF mutants examined, which accumulate much less otu protein. [2] Point mutations, which affect the alternate exon (a) and, hence, the 104-kD isoform, have been identified in both otu and otu. [3] Truncated otu proteins are expressed from two DIF alleles otu and otu, which presumably have normal alternate exons, yet these truncations have little discernible effect on cyst formation or differentiation. Moreover, otu and otu presumably produce perfectly functional 98-kD isoform and generate fertile flies when crossed with otu or otu, which can provide otu function through differentiation. These data in conjunction with the evidence that the 104-kD isoform is the predominant otu protein in undifferentiated cystocytes suggest that this isoform plays an important role in controlling the early mitotic divisions of oogenesis. Although the larger isoform appears to be important for controlling these early divisions and generating normal cysts, it is not absolutely required for cystocyte differentiation, because otu lacks this isoform entirely and yet produces some differentiated chambers. Perhaps high levels of the 98-kD isoform can promote cystocyte differentiation inefficiently.

Our present hypothesis is that the 104-kD isoform is expressed early in oogenesis and is required for proper control of germ cell divisions. The onset of differentiation results in an alternative splice such that the 98-kD isoform is expressed in stages subsequent to differentiation and that this isoform accumulates progressively in germ cell cytoplasm through stage 11, except in oocytes where it is rapidly degraded at the beginning of stage 11. These latter stages of oogenesis presumably require high levels of otu protein, as low levels result in the termination of development.

How might otu function?

Proteins from otu appear to play an important role in at least three different processes of oogenesis: stem cell proliferation, control of germ cell divisions, and degeneration and collapse of the nurse cells. In light of the
distribution of otu protein in ovaries [i.e., in germ-cell cytoplasm] we think the most likely function of otu is in controlling cytoskeletal reorganizations. The high level of otu protein in ovaries suggests the possibility that these proteins are integral components of specific cytoskeletal structures. However, otu proteins could also affect cytoskeletal reorganization by a number of alternatives.

Two studies support the idea that otu proteins perform a cytoskeletal role. The characteristic branched structure of 16-cell cysts is thought to be generated by a structure known as a polyfusome, which occupies the ring canals of the cyst [Maziarz 1913; Koch and King 1969; Mahowald and Strassheim 1970; Mahowald 1971; Teller 1975; King and Storto 1988]. Examination by electron microscopy and reconstruction of serial sections of germaria from the DIF mutant otu revealed that this mutant could not sustain a series of arrested cleavages and that its fusomal material contained ultrastructural differences from normal polyfusomes [Storto and King 1989]. It was suggested that otu gene products are involved in the organization and maintenance of ring canals through stabilization of the polyfusome, and if differentiation of cystocytes is linked to appropriate cyst differentiation of cystocytes then a disruption of the polyfusome could result in continued cell division and a tumorous phenotype. Nurse cell cytoplasmic transport is arrested in the DIF mutant otu, and this mutant lacks a network of actin microfilaments in nurse cells [Storto and King 1988], which are proposed to provide contractile forces that transport nurse cell cytoplasm into the oocyte through the ring canals [Gutzzeit 1986]. Interestingly, rapid loss of otu immunostaining in oocytes of normal egg chambers is coincident with development of actin microfilaments in nurse cells and the cytoplasmic transport process. If otu gene products perform a role in organizing the microfilament network perhaps degradation of otu protein in oocytes is required to prevent similar networks from forming in oocytes.

Although these cytoskeletal anomalies cannot account for the entire spectrum of otu mutant phenotypes, they provide insight into the function of otu proteins during oogenesis and a direction for studies of protein interactions with otu gene products.

Materials and methods

Antibody generation and purification

Production of anti-otu(253-671) antiserum has been described previously [Steinhauer et al. 1989]. Anti-otu(670-811) antiserum was produced by inserting a 3' EcoRI restriction fragment of cDNA 3-2 (nucleotides 4161-5777 excluding the intron, Steinhauser et al. 1989) into the pWR590 expression vector [Guo et al. 1984]. The lacZ-otu fusion construct was used to transform E. coli MV1184 cells. The expressed 90-kD fusion protein was partially purified by insoluble aggregation [Williams et al. 1982; Rio et al. 1986] and then gel purified by SDS-PAGE. Protein bands were visualized by Coomassie staining in water, excised, electroeluted, and precipitated with 8 vol of ethanol. Precipitated protein (50-200 |xg) was resuspended in a buffer containing 50 mM Tris-HCl [pH 8.0], 0.2% SDS, and 0.3 M NaCl, emulsified in an equal volume of Freund's complete adjuvant, and injected in multiple subcutaneous sites of rabbits. Rabbits were boosted at 3-week intervals with protein prepared as described above, emulsified in an equal volume of Freund's incomplete adjuvant.

Affinity columns were prepared with both fusion proteins by coupling to Affi-gel 10 [1 mg of protein/ml of gel], following the manufacturer's specifications [Bio-Rad]. Coupling buffer was 0.1 M NaHCO3 [pH 8.5], 0.3 M NaCl, and when required, 0.2% SDS was included to keep the fusion protein in solution. Addition of SDS in the coupling buffer did not interfere with coupling. High-titer sera were affinity purified on a lacZ-otu(670-811) fusion protein-affinity column following removal of antibody bodies against β-galactosidase on a lacZ-otu(253-671) fusion protein column. Loadings, washes, and elutions followed the procedures described by Robbins et al. [1984].

Sequence analysis

cDNA 4-1 was excised from λgt11 [Huynh et al. 1985] by EcoRI digestion and inserted into EcoRI-digested M13mp8 [Messing 1983]. The cDNA consists of two EcoRI fragments resulting from what appears to be a single-base change at nucleotide 2516 [Steinhauer et al. 1989], creating an additional EcoRI site. Nucleotide sequences were determined by the dideoxy chain-termination method [Sanger et al. 1977] on M13 single-stranded phage templates. The complete sequence of the alternate cDNA was determined on one strand by using oligonucleotide primers designed from the published sequence [Steinhauer et al. 1989]. Sequences were assembled and analyzed with the University of Wisconsin Genetics Computer Group programs [Devereux et al. 1984].

Western analysis

Ovaries were dissected from 2- to 6-day-old females that had been fed on yeast paste for 2 days at 18°C. Ovaries were homogenized in buffer containing 50 mM Tris-HCl [pH 7.5], 3 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM N-ethylmaleimide, 100 |xM leupeptin, 10 |xM pepstatin, 3 trypsin inhibitor units [TIU] of apronin/ml, and 100 |xg/ml PMSF and centrifuged at 13,000g for 3 min. Protein concentrations of the supernatants were determined by the method of Bradford [1976]. Protein recoveries were typically 5–10 |xg/ovary from Canton-S or otu DIF alleles and 0.5–1 |xg/ovary from otu ONC alleles.

Samples containing 10–20 |xg of protein were mixed with equal volumes of 2x sample buffer (Laemmli 1970) and fractionated on 10% SDS-polyacrylamide gels [1.5 mm]. Proteins were blotted to nitrocellulose overnight by electrophoretic transfer [Towbin et al. 1979]. Efficiency of transfer was estimated by transfer of prestained molecular mass markers [Bio-Rad]. Blots were blocked for 1 hr with 1% BSA [Promega] in TBST [10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween-20] followed by primary antibody incubation in TBST. Incubation times varied with the antibodies used [anti-otu(253-671), 1 hr, anti-otu(670-811), 3 hr]. After washing four times for 10 min each in TBST, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody in TBST for 1 hr [Boehringer Mannheim, 1:5000]. Blots were washed four times for 10 min each in TBST and developed with BCIP and nitroblue tetrazolium [Bio-Rad] following the manufacturer's specifications.

PCR amplification

Genomic DNA for PCR amplification was prepared from mutant flies by mashing three flies with a toothpick in 50 |xg of a

Analysis of protein expression from otu
buffer containing 10 mM Tris-HCl (pH 8.2), 1 mM EDTA, 25 mM NaCl, and 200 μg/ml of proteinase K. Homogenates were heated to 95°C for 15 min and, after addition of 2 μl of 10 mg/ml PMSF, reheated to 65°C for an additional 15 min. Homogenates were then centrifuged to pellet the debris, and the supernatant was removed. A 10-μl aliquot of this supernatant was used in each PCR reaction.

PCR [Saiki et al. 1988] was performed with the GeneAmp kit [Perkin-Elmer Cetus] following the manufacturer’s specifications. Reactions were cycled 30 times [94°C for 1 min, 55°C for 1 min, 72°C for 1 min], and resulting fragments were digested with EcoRI and HindIII, gel purified, and inserted into EcoRI/ HindIII-digested M13mp8 and M13mp9. Primers for amplifying across the alternate exon were ATCAAGCTT-2643-2662 [Fig. 3] and AGCGATTC-3050-3031 [Fig. 3], which amplified a 408-bp genomic fragment from otu15 and otu16. The primers used to find the nonsense mutations in otu5 and otu14 were GCTAAGCTT-3947-3966 and 4197-4178 [for sequence, see Steinhauser et al. 1989]. In this case, the internal EcoRI restriction site just upstream of the 5’ primer, along with the HindIII site in the 5’ primer, was used to clone the amplified 251-bp genomic fragment. Use of the internal EcoRI site resulted in the insertion of 219 bp of genomic DNA from otu15 and otu14 into EcoRI/HindIII-digested M13mp8 and M13mp9. All sequencing reactions were performed as described above.

Tissue preparation and immunoperoxidase staining

Prior to immunostaining, control antisera were generated by removal of otu-specific antibodies on fusion protein-affinity columns. Two hundred microliters of anti-otu(670-811) antiserum was loaded onto a lacZ-otu(253-671) and a lacZ-otu(670-811) affinity column, and washed with 10 ml of PBS containing 1% BSA, and the flowthrough was recycled over the column four times. The final flowthrough from the lacZ-otu(253-671) column was the experimental sera, whereas the flowthrough from the lacZ-otu(670-811) column served as the control sera. Similarly, 80 μl of anti-otu(253-671) antiserum was loaded on affinity columns and processed as described above. Flowthrough from the lacZ-otu(670-811) column was the experimental sera, and flowthrough from the lacZ-otu(253-671) column was the control sera.

Ovaries were dissected from 2- to 6-day-old females that had been fed on yeast paste for 2 days at 18°C. Ovaries were frozen directly in optimally controlled temperature (OCT) compound [Miles, Inc.] and sectioned (6 μm) on a cryotome. Sections were mounted on glass slides coated with poly-L-lysine and air-dried at room temperature. Sections were fixed with formalin for 30 min, washed twice for 5 min in PBS [10 mM sodium phosphate (pH 7.4), 150 mM NaCl], and treated with 1% H2O2 in PBS for 10 min. After two rinses with PBS and a 10-min incubation with 2% lamb serum in PBS, sections were incubated for 24–48 hr at 4°C with dilutions [anti-otu(253-671) 1:40; anti-otu(670-811) 1:4] of the experimental and the control antisera in PBS containing 1% BSA. Immunoreactivity was localized by the avidin-biotin peroxidase complex technique (Hsu et al. 1981), using dianinobenzidine for formation of the reaction product.

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References

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Brown, E.H. and R.C. King. 1964. Studies on events resulting in formation of an egg chamber in Drosophila melanogaster. Growth 28: 41–81.

Carroll, S.B. and M.P. Scott. 1985. Localization of the fushi tarazu protein during Drosophila embryogenesis. Cell 43: 47–57.

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.

Cummings, M.R. and R.C. King. 1969. The cytology of the vitelligenic stages of oogenesis in Drosophila melanogaster. I. General staging characteristics. J. Morphol. 128: 427–440.

Davis, S.I. and M. Metcalf. 1968. A re-evaluation of the duration of egg chamber stages in oogenesis of Drosophila melanogaster. Drosophila Inform Serv. 43: 122–123.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12: 387–395.

Digan, M.E. 1980. “A genetic analysis of chorion morphogenesis in Drosophila melanogaster.” Ph.D. thesis, Indiana University, Bloomington, IN.

Driever, W. and C. Nüsslein-Volhard. 1988. A gradient of bicoid protein in Drosophila embryos. Cell 54: 263–269.

Gans, M., C. Audit, and M. Masson. 1975. Isolation and characterization of sex-linked female-stereile mutants in Drosophila melanogaster. Genetics 81: 683–704.

Gaul, U., E. Seifert, R. Schuh, and H. Jäckle. 1987. Analysis of krüppel protein distribution during early Drosophila development reveals posttranslational regulation. Cell 639–647.

Guo, L., P.P. Stepfen, J.Y. Tso, R. Brousseau, S. Narang, D.Y. Thomas, and R. Wu. 1984. Synthesis of human insulin gene. VIII. Construction of expression vectors for fused proinsulin production in Escherichia coli. Gene 29: 251–254.

Gutzeit, H.O. 1986. The role of microfilaments in cytoplasmic streaming in Drosophila follicles. J. Cell Sci. 80: 159–169.

Hsu, S.M., L. Raine, and H. Fanger. 1981. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody [PAP] procedures. J. Histochem. Cytochem. 29: 577–580.

Huynh, T.V., R.A. Young, and R.W. Davis. 1985. Constructing and screening cDNA libraries in X.gtlO and X.gtl1. In DNA cloning techniques: A practical approach [ed. D. Glover], pp. 49–78. IRL Press, Oxford.

Kinderman, N.B. and R.C. King. 1973. Oogenesis in Drosophila virilis. I. Interactions between the ring canal rims and the nucleus of the oocyte. Biol. Bull. 144: 331–354.

King, R.C. 1970. Ovarian development in Drosophila melanogaster. Academic Press, New York.

———. 1979. Aberrant fusomes in the ovarian cystocytes of the fs(1)231 mutant of Drosophila melanogaster Meigen (Diptera: Drosophilidae). Int. J. Insect Morphol. Embryol. 8: 297–309.

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., A.C. Rubinson, and R.F. Smith. 1956. Oogenesis in adult Drosophila melanogaster. Growth 20: 121–157.
King, R.C., D. Mohler, S.F. Riley, P.D. Storto, and P.S. Nicolazzo. 1986. Complementation between alleles at the ovarian tumor locus of Drosophila melanogaster. Dev. Genet. 7: 1–20.
Koch, E.A. and R.C. King. 1969. Further studies on the ring canal system of the ovarian cystocytes of Drosophila melanogaster. Z. Zellforsch. 102: 129–152.
Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
Mahowald, A.P. 1971. The formation of ring canals by cell furrows in Drosophila. Z. Zellforsch. Mikrosk. Anat. 118: 162–167.
Mohler, J.D. 1977. Developmental genetics of the Drosophila egg. I. Identification of 59 sex-linked cistrons with maternal effects on embryonic development. Genetics 85: 259–272.
Mulligan, P.K. and E.M. Rasch. 1985. Determination of DNA content in the nurse and follicle cells from wild-type and mutant Drosophila melanogaster by DNA-Fuelgen cytophotometry. Histochemistry 82: 233–247.
Moynahan, P.P., J.D. Mohler, and L.J. Kalilayan. 1988. Molecular localization and developmental expression of the otu locus of Drosophila melanogaster. Mol. Cell. Biol. 8: 1481–1488.
Prost, E., F. Deryckere, C. Roos, M. Haenlin, V. Pantesco, and E. Mohier. 1988. Role of the oocyte nucleus in determination of the dorsoventral polarity of Drosophila as revealed by molecular analysis of the K10 gene. Genes & Dev. 2: 891–900.
Rio, D.C., F.A. Laski, and G.M. Rubin. 1986. Identification and immunochemical analysis of biologically active Drosophila P element transposase. Cell 44: 21–32.
Robbins, A., W.S. Dynan, A. Greenleaf, and R. Tjian. 1984. Affinity-purified antibody as a probe of RNA polymerase II subunit structure. J. Mol. Biol. Genet. 2: 343–353.
Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. 74: 5463–5467.
Steinhauer, W.R., R.C. Walsh, and L.J. Kalilayan. 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. Wilhelm Roux's Arch. Dev. Biol. 196: 210–221.

—- 1988. Multiplicity of functions for the otu gene products during Drosophila oogenesis. Dev. Genet. 9: 91–120.
—- 1989. The role of polyfusomes in generating branched chains of cystocytes during Drosophila oogenesis. Dev. Genet. 10: 70–86.
Telfer, W.H. 1975. Development and physiology of the oocyte-nurse cell syncytium. Adv. Insect Physiol. 11: 223–319.
Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. 76: 4350–4354.
Williams, D.C., R.M. Van Frank, W.L. Muth, and J.P. Burnett. 1982. Cytoplasmic inclusion bodies in Escherichia coli producing biosynthetic human insulin proteins. Science 215: 687–689.
A specific ovarian tumor protein isoform is required for efficient differentiation of germ cells in Drosophila oogenesis.

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