The Drosophila gene fs(2)cup interacts with otu to define a cytoplasmic pathway required for the structure and function of germ-line chromosomes

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SUMMARY

The Drosophila ovarian tumor gene (otu) encodes cytoplasmic proteins that are required in germ-line cells for cyst formation, nurse cell chromosome structure and egg maturation. We have analyzed a gene, fs(2)cup, that participates in many of the same processes and interacts with otu genetically. Both nurse cell and oocyte chromosomes require cup to attain a normal morphology. In addition, the gene is needed for the oocyte to grow normally by taking up materials transported from the nurse cells. The gene encodes a 1132-amino-acid protein containing a putative membrane-spanning domain. Cup protein (but not cup RNA) is transported selectively into the oocyte in germlarial cysts, like the p104 Otu protein. It is strongly associated with large structures in the cytoplasm and perinuclear region of nurse cells and, like Otu, moves to the periphery of these cells in stages 9-10. Moreover, cup mutations dominantly disrupt meiotic chromosome segregation. We propose that cup, otu and another interacting gene, fs(2)B, take part in a common cytoplasmic pathway with multiple functions during oogenesis.

Key words: Drosophila, ovarian tumor gene, otu, cup, chromosome, germ line, nurse cell, egg maturation, oocyte

INTRODUCTION

Eggs are produced rapidly in the adult Drosophila ovary due to the activity of specialized accessory cells (reviewed in Spradling, 1993). In the gerarium located at the tip of each ovariole (Fig. 1a), germ-line cells containing a special cytoplasmic structure, known as the fusome, divide synchronously four times to form germ-line cysts containing 16 interconnected cells. 15 of the cells differentiate as nurse cells, which synthesize and transport products required for the development of the remaining cell, the synthetically inactive oocyte (Fig. 1a). In addition, follicle cells surround growing 16-cell cysts while they still reside within the gerarium. Eventually, cysts and their associated follicle cells bud off as discrete units called egg chambers, each separated from adjacent chambers by follicular stalks.

Germ-line cell chromosomes become highly adapted for the specialized functions they perform during oogenesis. Oocyte chromosomes undergo synapsis and recombination during the early stages of cyst development. Then the nucleus condenses into a synthetically inactive karyosome around stage 3 (see Fig. 1a for stages), and remains in meiotic prophase until just before the oocyte matures. Nurse cell chromosomes also undergo visible changes in structure as egg chambers develop (Hsu and Hansen, 1953; King, 1970; Hammond and Laird, 1985). The banded polytene chromosomes characteristic of young nurse cells progressively disperse, and large, dispersed nucleoli form. These changes in nurse cell chromosome organization have been postulated to facilitate the high synthetic levels of ribosomes and other components required for rapid oocyte formation (Spradling, 1993).

Several genes are required for normal nurse cell chromosome development and to sustain normal oocyte growth, including fs(2)B (King et al., 1957), suppressor of Hairy-wing (su(Hw), Klug et al., 1968), ovarian tumor (otu, King et al., 1981), D-elt (Schultz et al., 1993) and string-of pearls (Crampton and Laski, 1994). The role played by otu is particularly interesting, since nurse cells bearing certain otu alleles frequently develop large polytene chromosomes (King and Storto, 1988) and are unable to support a normal rate of oocyte growth.

The otu gene has long been thought to play a key role in cyst development and growth based on the analysis of three classes of alleles (Storto and King, 1988). Germ-line cells are absent in quiescent (QUI) alleles, but overproliferate to form benign tumors in oncogenic (ONC) alleles. In differentiating (DIF) alleles, nurse cells display abnormal chromosome morphology, fail to growth normally and do not fully transfer their contents to the oocyte. otu encodes a 104 kDa cytoplasmic protein sufficient for gene function as well as a 98 kDa isoform of uncertain function (Rodesch et al., 1995; Sass et al., 1995). At least some germ cells in otu tumors fail to undergo proper female sex determination (Pauli et al., 1993), but it remains unclear whether this is a direct effect (Bae et al., 1994; Horabin et al., 1995). Otu has also been proposed to act via the fusome (King and Storto, 1988), the cytoskeleton (Sass et al., 1995) and in association with microtubules (Tirronen et al., 1995).

We have characterized a new gene, fs(2)cup, that affects many of the same processes as otu. cup encodes a cytoplasmic
protein that is expressed in a similar pattern to Otu p104, and interacts genetically with the p104-specific otu<sup>11</sup> allele. Our results imply that these genes participate in a specialized cytoplasmic pathway required for egg chamber formation and growth. We speculate that it may involve the trafficking of microtubule-associated vesicles.

**MATERIALS AND METHODS**

**Drosophila stocks and crosses**

Flies were raised on standard food at 22-25°C except as indicated. Genetic markers and balancer chromosomes are described in Flybase (1993). cup<sup>1355</sup>, cup<sup>4506</sup> and cup<sup>6890</sup> were recovered in a single-P element mutagenesis screen (Karpen and Spradling, 1992). Derivatives of cup<sup>4506</sup> and cup<sup>1355</sup> were generated by inducing transposition of the P[<i>lacZ, ry</i>] element in the male germ line. EMS-induced alleles of cup were provided by T. Schüpbach. Alleles of otu (see King and Storto, 1988) were obtained from A. Mahowald and from the Bloomington Stock Center. Strains bearing fs(2)B were kindly provided by the Bowling Green Stock Center. For non-disjunction tests, progeny stocks were backcrossed to parental otu and cup chromosomes to confirm their genotypes.

**Microscopy**

Whole ovaries were dissected, fixed and mounted in 50% glycerol containing 1 μg/ml DAPI as described previously (Lin and Spradling, 1993). Relative egg chamber volumes were estimated by using the program NIH Image.

**Molecular analysis**

Genomic DNA flanking the insertion in cup<sup>4506</sup> was isolated by plasmid rescue and used to probe a genomic library (Maniatis et al., 1978). cDNAs were isolated from an ovarian library (Stroumbakis et al., 1994). In situ hybridization of digoxigenin-labeled sense and antisense cDNA probes was carried out using the Genius labeling kit (Boehringer) by the method of Tautz and Peifle with modifications (Suter and Steward, 1991).

**Antibody production and western analysis**

Bacterially expressed Cup protein for immunization was produced using the 6x his tag system (QIAExpress, Qiagen). Two regions of the predicted Cup protein (cDNA nucleotides 758-1655 and 1655-2729) were independently fused in frame to N-terminal histidine tags. Recombinant protein from bacterial lysates affinity-purified over a Ni-NTA column (Qiagen) was used as immunogen. Polyclonal rat antisera were produced by HRP, Inc. Ovary extracts from wild-type and mutant females run on a 7% SDS-PAGE gel were blotted to nitrocellulose for western analysis. Primary anti-Cup antibodies were detected using secondary horseradish peroxidase-conjugated goat anti-rat IgG (Amersham) and chemiluminescent staining (ECL, Amersham). Antibody staining of ovaries was carried out as described in Lin et al. (1994).

**RESULTS**

**Identification of fs(2)cup, a gene that disrupts nurse cell chromosome structure**

Changes in chromatin structure that accompany wild-type nurse cell development are readily observed following DAPI staining of whole ovarioles (Fig. 1b). Nurse cells initially contain banded polytene chromosomes that can be resolved by squashing (Hsu and Hansen, 1953). During stages 4-5 (Fig. 1b,
The strongest class I alleles produce enlarged and misshapen egg chambers to arrest prior to vitellogenesis. Alleles that also contain insertions in the 27B region, such as fs(2)01355 and rev, revealed that effects of cup alleles on oogenesis viability or male fertility. Effects of cup alleles on oogenesis

The cup alleles can be grouped into three general classes based on their stage of arrest (Table 1). Class I, the largest set of alleles, causes egg chambers to arrest prior to vitellogenesis. The strongest class I alleles produce enlarged and misshapen germaria from which egg chambers do not always bud normally (Fig. 2a). Frequently two or more chambers remain tightly associated, and the nurse cells fail to grow past early stages. Commonly, as in cup4506, egg chambers appear almost normal until stage 5, fail to decondense nurse cell chromatin, and subsequently degenerate by the time they reach the size of stage 7-8 egg chambers.

Egg chambers from class II females grow larger than class I chambers, taking up yolk and sometimes supporting follicle cell migration. However, nurse cell nuclei display abnormal chromatin configurations that can be easily visualized due to their large size. Some class II alleles retain the five-blob morphology characteristic of normal stage 5 egg chambers and of class I alleles (Fig. 2b), but others appear to contain 8-16 small polytene chromosomes in place of each blob (Fig. 2c,f). The exact borders of the domains are not sufficiently distinct to determine the exact number of these ‘sub-polytene’ chromosomes, however, and the chromosomal organization of individual nuclei frequently varies within a single egg chamber.

Females from the weakest group of cup alleles, class III, produce defective mature eggs characteristically shaped like cups. Egg chambers that will develop into cup-shaped eggs have normal proportions during early stages, but during stages 9 and 10 their oocytes reach only 1/4 to 1/2 the size of corresponding wild-type oocytes, and occupy no more than 33% instead of 50% of the egg chamber. The dwarf oocytes are not caused by a reduction in growth per se; indeed, some cup egg chambers become larger than wild type prior to nurse cell breakdown, despite their small oocytes, and contain extra follicle cells (Fig. 2i). The decreased size of the cup oocyte relative to its nurse cells suggests that the transport of materials from the nurse cells into the oocyte is reduced during stages 9 and 10. Nurse cell chromatin also remains abnormally condensed except in cup1355 and cup6890, which have normal chromatin morphology.

The cup-shaped chorions characteristic of class III alleles result from changes in follicle cell migration that may be caused indirectly by the undersized oocytes. Normally, at stage 9 most of the follicle cells migrate posteriorly onto the oocyte surface (Fig. 2g). Later, some of these cells migrate centripetally

### Table 1. Properties of selected cup alleles

<table>
<thead>
<tr>
<th>Class</th>
<th>Allele</th>
<th>Stage of arrest</th>
<th>Nurse cell nuclei</th>
<th>Similar alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>R4^4</td>
<td>S5</td>
<td>Little DNA</td>
<td>8^b, 17^b</td>
</tr>
<tr>
<td></td>
<td>4506^6</td>
<td>S5-6</td>
<td>Like normal S5</td>
<td>3^b, 6^b, 22^b, 33^b</td>
</tr>
<tr>
<td></td>
<td>20^b</td>
<td>S5-6</td>
<td>Tight condensation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16^b</td>
<td>S6-7</td>
<td>5-blob, variable</td>
<td>28^b</td>
</tr>
<tr>
<td>II</td>
<td>13^b</td>
<td>S7-8</td>
<td>5-blob, variable ploidy</td>
<td>31^b</td>
</tr>
<tr>
<td></td>
<td>15^b</td>
<td>S7-8</td>
<td>Decondensed, variable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32^b</td>
<td>S10</td>
<td>Condensed oligotene, high ploidy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4^b</td>
<td>S10</td>
<td>Polytenes, high ploidy</td>
<td>5-blob</td>
</tr>
<tr>
<td></td>
<td>1^b</td>
<td>S9-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1355^a</td>
<td>S14</td>
<td>Normal</td>
<td>6890^a, 26^b</td>
</tr>
<tr>
<td></td>
<td>24^b</td>
<td>S14</td>
<td>Variable condensation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21^b</td>
<td>S14, weakly fertile</td>
<td>Variable condensation</td>
<td></td>
</tr>
</tbody>
</table>

Cup alleles are grouped into three major classes, as described in the text.

Sources: a, Karpen and Spradling (1992); b, Schüpbach and Wieschaus (1986); c, this study.

The order of the alleles reflects severity, as indicated by the oldest stage typically reached by egg chambers prior to degenerating. The morphology of nurse cell nuclear chromatin in the oldest chambers is listed.

Similar alleles: other cup alleles whose phenotype does not appear significantly different from the described prototype allele.
between the nurse cells and the oocyte, where they will eventually secrete the anterior end of the eggshell (Fig. 2h). *cup* oocytes may be too small to accommodate all the migrating follicle cells; many cells are observed to remain in contact with the four posterior nurse cells (Fig. 2i). A centripetal migration never occurs. Eventually, a chorion layer is secreted over the oocyte and posterior nurse cells by the overlying follicle cells, leaving the anterior end open, like a cup (Fig. 2j). Dorsal appendage-secreting follicle cells remain near their site of induction by the oocyte nucleus.

With a few possible exceptions, *cup* alleles behave as a continuous allelic series. Crosses using representative alleles from each phenotypic class produced progeny whose phenotype was intermediate between the parents, suggesting that these individual alleles produce different quantitative levels of *cup* function. For example, when a class I allele causing early arrest (*cup^R4^*) was crossed to a class III allele (*cup^1355^*), the ovarian arrest and chromosomal phenotype of the transheterozygote resembled that seen in class II chambers (Fig. 2f). Moreover, when stronger alleles from within class I and class III are used to make the combinations, egg chambers arrest growth earlier than when weaker alleles are combined.

Two alleles appeared to be distinctive. *cup^4^* (class II) chambers frequently contain large banded polytene chromosomes in their nurse cells (Fig. 2e). These chambers develop as far as stage 9 or 10 and the chromosomes achieve high ploidy levels. This behavior is similar to several *otu* alleles that produce polytene chromosomes of sufficient quality for cytological analysis (King and Storto, 1988). *cup^20^* (class I) chromosomes also remain tightly condensed, as in stage 4, with some evidence of a banding pattern. However, chromosomal organization within the nucleus is unique; the individual chromosomes traverse a uniform spiral path around the inside of the nuclear membrane (Fig. 2d). These phenotypes are not observed in other *cup* alleles or allelic combinations and may reveal an additional function of the Cup protein. The lack of a deficiency for the 27B region prevented further tests of the nature of these alleles.

**Cloning the *cup* gene**

Genomic DNA flanking the *cup^4506^* insertion was isolated by plasmid rescue and used as an entry point to the *cup* locus. Within a 30 kb genomic region surrounding the insertion site, northern analysis identified a 4.1 kb poly(A)-containing RNA that is detected only in the ovary and in 0-2 hour embryos, but not in later embryos or other tissues (Fig. 3b). The 4.1 kb RNA is severely reduced in the ovaries of *cup* mutant females (Fig. 3b). In the testes, the 4.1 kb transcript is absent, but two male-specific transcripts of 4.3 and 3.8 kb are detected. An apparently full-length 4.1 kb cDNA was isolated from an ovarian cDNA library and used to map the position of the trans-
Fig. 3. Molecular structure of cup. (a) A restriction map of the 11 kb genomic region that includes the cup locus and P element insertion sites is shown. The 5' end of the 4.1 kb cDNA is indicated by an arrow. The cup1355 insertion lies within untranslated exon 1; the cup4506 and cup890 insertions are presumed to reside within introns since their flanks do not match the cDNA sequence, but the exact number and position of introns was not fully determined. (b) Northern blot of poly(A)+ RNA hybridized with cup cDNA; tubulin reprobe is shown below. Lanes: 1, 0-4 hour embryos; 2, 5-8 hour embryos; 3, 8-24 hour embryos; 4, wild-type testes; 5, wild-type ovaries; 6, cup4506 ovaries; 7, cup890 ovaries. (c) Schematic map of the cup cDNA. (d) Nucleotide and amino acid sequence of the 4.1 kb cDNA. The position of the cup1355 insertion is indicated by a triangle.

(residues 116-136) that lies adjacent to a small predicted coiled coil region (residues 135-159); no signal sequence was detected. The remainder of the protein contains multiple asparagine- and glutamine-rich regions (Fig. 3d). Database searches using BLAST and FASTA detected no strong homology to any previously identified genes.

Searches with two pattern-based programs, BLOCKS (Henikoff and Henikoff, 1994) and FASTA-PAT (Ladunga et al., 1996), detected weak similarities between Cup and several proteins involved in vesicle trafficking and/or microtubule binding. The BLOCKS program found a match between Cup and the vertebrate microtubule-associated protein MAP1B that was better than expected in 99% of comparisons between the randomized sequences. The strongest similarity is found between amino acids 190-212 of Cup and amino acids 2446-2468 that form the COOH terminus of MAP1B. The FASTA-PAT program matched Cup to a pattern derived from the yeast Uso1 protein and the related vertebrate p115 gene (Sapperstein et al., 1995) with a probability value given as 10^-29. (Simple FASTA alignment of Cup and Uso1 shows that approximately 14% of residues are identical over the whole protein.) Both Uso1p and p115 are known to be involved in the reassembly of ER and Golgi vesicles following mitosis. Scores lower than 10^-26 were also recorded for three other proteins: gp360 macroglgin, a Golgi-associated protein (Seeig et al., 1994), nestin, a nuclear intermediate filament protein (Kachinsky et al., 1994), and the CLIP170 vesicle-microtubule linker protein (Pierre et al., 1992). The significance of these similarities remains uncertain.

Expression of cup gene products

To learn where cup is transcribed during oogenesis, the 4.1 kb
cDNA was hybridized in situ to wild-type ovaries. *cup* RNA is present in germ-line cells throughout pre-vitellogenic development, but is not detected in the somatic follicle cells (Fig. 4a). The RNA is present in region 1 of the germarium, where it is detectable in stem cells, cystoblasts and dividing cysts (Fig. 4c). Steady state RNA levels decrease in region 2a, rise again in region 2b, and peak around stage 3 or 4. Subsequently, RNA levels decline and reach undetectable levels by stage 8. A second round of expression begins during stage 10 (Fig. 4b) and continues through stage 14. The transcript is not differentially localized within the germ line at any stage. None of the strong alleles abolished all *cup* RNA expression (Fig. 4d and data not shown).

Polyclonal antibodies were raised against two independent domains of the Cup protein. Antisera against both domains produce similar results when used to analyze ovarian extracts on western blots. Both sera detect a single predominant band of approximately 150 kDa that is abundant in wild-type ovaries, but greatly reduced in *cup* mutant females (Fig. 5a). This is similar in size to the 130 kDa product predicted by the 4.1 kb cDNA. A second smaller band of 75 kDa reacts more weakly and variably with all *cup* sera tested, but this protein is unaffected by mutants and is presumed to be encoded by a separate locus. Testes contain very little of the 150 kDa protein; the anti-Cup antibodies instead detect bands of approximately 180, 110 and 45 kDa (Fig. 5a). This suggests that the testis-specific *cup* RNAs (Fig. 3b) encode non-identical but antigenically related Cup proteins.

The protein products of the EMS- and P-derived *cup* alleles were examined by western analysis. All but one of the *cup* alleles affect the quantity, rather than size of the 150 kDa protein, indicating that these alleles are not the products of premature termination. None of the strong *cup* alleles completely eliminates all immunoreactive protein (Fig. 5a, lanes 3-7 and data not shown), suggesting that null alleles have not been recovered. The level of Cup protein detected in these experiments correlates well with the genetic strength of the allele in question: weak alleles such as *cup*1355 and *cup*6890 (Fig. 5a, lanes 4 and 5) produce much more of the 150 kDa Cup protein than strong alleles such as *cup*16 and *cup*20 (Fig. 5a, lanes 6 and 7). One allele, *cup*1, produced a smaller Cup protein of about 135 kDa (Fig. 5b), further confirming that the gene analyzed corresponds to *cup*.

![Fig. 4](image) Expression of *cup* RNA in ovaries. In situ hybridization of antisense-labelled *cup* cDNA to whole ovaries. (a) Wild-type ovariole, through stage 9. (b) Stage 10b, wild-type ovariole. (c) Germarium of wild-type ovariole; stages of germarial development are indicated. (d) Germarium of *cup*Δ ovariole. Scale bars, 20 μm.

**Immunolocalization of Cup protein**

Immunofluorescent antibody staining was used to determine the expression pattern and subcellular localization of the Cup protein. Beginning with the stem cells, Cup protein is found in the cytoplasm of all germ-line cells (Fig. 6a,b), but was never detected inside either nurse cell or oocyte nuclei. Staining of early embryos confirmed that Cup protein is maternally deposited in the egg (Fig. 6g). The protein is abundant and uniformly distributed in the cytoplasm of all cleavage stage embryos through stage 9, after which signal intensity declines. Staining is effectively absent by gastrulation, and remains so throughout the remainder of embryogenesis.
The subcellular location and distribution of Cup protein within 16-cell cysts underwent marked changes during the course of oogenesis. Cup protein accumulates preferentially in the future oocyte within 16-cell cysts of the germarium. This enrichment begins very early: localization to a single cell is detectable in region 2a, prior to overt differentiation of the oocyte (Fig. 6a). Unlike most oocyte-enriched proteins such as Bic-D or Oskar, no corresponding enrichment of cup mRNA was observed (Fig. 4a). Cup protein may be selectively transported from the nurse cells and/or differentially translated or stabilized within the oocyte. The retention of relatively high levels of Cup protein in the nurse cells suggests that the protein may function in both cell types.

Cup protein continues to be selectively enriched in the oocyte until approximately stage 8, a time when egg chamber microtubules are extensively reorganized (Theurkauf et al., 1992). During stage 9 the nurse cells and oocyte contain similar amounts of Cup protein (Fig. 6d), and by stage 10 most of the protein lies in the nurse cells. Prior to stage 8 Cup forms a cap at the posterior of the oocyte; later much smaller amounts of protein are found at the oocyte surface and also persist in a small cap at the posterior.

The behavior of Cup protein in the nurse cells is particularly interesting. Cup accumulates almost exclusively in large aggregates, whose location varies as egg chambers develop (Fig. 6c,d). In early chambers, and especially around stage 4, Cup aggregates are found predominantly around the periphery of the nurse cell nuclei (Fig. 6b,c). After stage 4, Cup leaves the nuclear membrane and becomes dispersed throughout the nurse cell cytoplasm in large aggregates that eventually move toward the cellular periphery. By stage 10, Cup protein is localized almost exclusively in particulate structures along the subcortical surface of the nurse cells (Fig. 6e,f). The movement of Cup protein away from the nucleus corresponded in time to the sharp reduction in cup mRNA levels after stage 4, suggesting that a decrease in the rate of Cup synthesis might play a role in these changes.

Cup mutations drastically reduce the level of protein detectable by immunofluorescence microscopy using anti-Cup antibodies (data not shown). This was true even in the germarium, where the effects of mutations on the steady state level of cup RNA were weakest. In females homozygous for cup\(^1\), the one allele for which a physical change in the protein has been confirmed, residual protein was still localized to the oocyte (Fig. 6h).

**cup interacts with otu early in development**

To further analyze the role played by cup in oogenesis, we

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**Fig. 6.** Expression and localization of the Cup protein. Confocal microscopy of fluorescein-stained anti-Cup antibody in ovaries and embryos. (a and b) Germarium and early egg chambers: enrichment of the protein can be seen in the presumptive oocyte as early as region 2a. (c) Higher magnification of a stage 4 egg chamber, showing the ring of Cup aggregates around the nurse cell nuclei. (d) Stages 5, 7 and 9: the loss of oocyte enrichment and clearing of Cup protein from the internal regions of the nurse cells can be seen. (e) Stage 10b nurse cells, optically sectioned along the surface of the chamber. (f) Same chamber as in e, medial optical section. (g) Wild-type cleavage stage embryos, showing loss of Cup staining during blastoderm formation. (h) cup\(^1\) ovariole. A small amount of Cup protein remains visible in the oocyte, in large aggregates. (i) Dicephalic egg chamber from otu\(^1\)/+; cup\(^1\)/cup\(^1\) ovariole, showing Cup protein enriched in the centrally located oocyte. The higher apparent background staining in h and i is an artifact of microscopy conditions used to maximize detection of low levels of Cup protein.
looked for genetic interactions between cup and previously described genes with similar effects on development. Several observations suggested that cup and otu might interact. Late arresting alleles of otu affect condensation of the nurse cell chromosomes and partially prevent nurse cells from transferring their contents into the oocyte. Moreover, the distribution of Otu p98 and p104 parallel Cup expression: p104 becomes enriched in early oocytes and both proteins move toward the thirn their contents into the oocyte. The addition of one copy of otu to cup homozygotes produced an additional effect not seen in cup alleles alone: many of the egg chambers in otu/cup ovaries were dicyclic, with nurse cells positioned on both ends of a centrally located oocyte (Fig. 7d). The effect on egg chamber polarity varied depending upon the strength of the cup allele. In the combination otu/cup, 80% (36/45) of stage 9 or older egg chambers were dicyclic. Substitution of cup or otu for cup produced dicyclic chambers at a frequency of 41% (24/58) and 18% (19/106), respectively. Dicyclic chambers were never observed in any of the cup alleles (<0.1% for cup, 1% for other alleles) or in otu heterozygotes (<1%).

Immunofluorescent staining of dicyclic chambers indicates that Cup protein is enriched in the centrally located oocytes (Fig. 6i), suggesting that the disruption of normal chamber polarity does not affect the ability of Cup protein to preferentially accumulate in the oocyte.

We carried out western blotting and immunofluorescence labeling to determine if the suppression of cup sterility by otu involved a restoration of Cup protein. Cup ovaries with one copy of otu produce much more Cup than cup wild type for cup. (Fig. 5b: lanes 2 and 3). Similar results were obtained for otu and cup (data not shown). Much of the

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protein in these egg chambers is found in large aggregates in the oocyte (Fig. 6i). In both examples protein levels remain below wild type, and a novel, smaller band is detected that may represent an abnormal form of Cup. Thus \textit{otu\textsuperscript{11}} partially restores \textit{cup} function by increasing the amount and possibly altering the structure of Cup products.

\textbf{\textit{cup} interacts with \textit{fs(2)B}}

\textit{fs(2)B} is another female sterile mutation that arrests egg chamber development and produces nurse cells with polytene chromosomes. \textit{fs(2)B} is a dominant modifier of \textit{otu} \cite{King1981}, suggesting that these genes are related in function. We tested \textit{fs(2)B} for interaction with \textit{cup} alleles. In a \textit{cup\textsuperscript{+}} background, \textit{fs(2)B} homozygotes arrest early. Egg chambers develop to stage 14 only rarely and such eggs are not laid (Fig. 7e). By contrast, when the females are also heterozygous for \textit{cup\textsuperscript{4}}, \textit{fs(2)B} homozygotes are weakly fertile, with an average of one viable embryo per female \((n=19)\). Most of the mature egg chambers produced are not laid; although these resemble wild type and are covered with an apparently normal chorion, they contain abnormal DNA masses within the oocyte (Fig. 7f). \textit{fs(2)B} females are also weakly rescued to fertility by the addition of one copy of another class II allele, \textit{cup\textsuperscript{13}}, but not by the strong class I allele, \textit{cup\textsuperscript{04506}}. In all three cases ovarian development progresses further than in \textit{fs(2)B} homozygotes and the frequency of tumor formation is greatly reduced, but oogenesis remains abnormal with defects resembling those seen in \textit{fs(2)B} alone (Fig. 7f). Despite the relatively weak suppression by \textit{cup} of the \textit{fs(2)B} oogenesis defects, the production of viable progeny suggests that the interaction is significant. These results, in addition to the previous evidence that \textit{some \textit{fs(2)B} and \textit{otu} alleles interact in their effects on nurse cell chromosome structure, strongly suggest that all three genes affect common processes and may lie in a single pathway.

\textbf{\textit{Cup} mutations disrupt meiotic chromosome segregation}

In the course of studying the interactions between \textit{cup} and \textit{otu}, another effect of \textit{cup} mutations became apparent. Females of the genotype \textit{otu\textsuperscript{11}/FM6; cup\textsuperscript{15}/CyO} were observed to produce a high frequency of exceptional progeny displaying non-Mendelian inheritance of \textit{X} chromosomal markers. The production of patroclinous sons, (sterile \textit{XO} males with paternally derived \textit{X} chromosome markers), as well as matroclinous daughters (presumptive \textit{XXY} females with maternal markers), suggests a high rate of failure of normal \textit{X} chromosome disjunction during meiosis in the mother.

Meiotic non-disjunction in these females could result from the reduction in \textit{cup}, \textit{otu} or both. In addition, achiasmatic balancer chromosomes contribute to chromosome loss by preventing the recombination events that physically link the homologues during meiotic prophase I. To determine the relative contribution of these factors in causing nondisjunction, we crossed \textit{otu\textsuperscript{11}/FM6; cup\textsuperscript{15}/CyO} females to \textit{y; Sco/CyO} males. Independent segregation of the \textit{otu}, \textit{cup} and balancer chromosomes results in six classes of related females, which were then tested for fidelity of \textit{X} chromosome transmission. The results are summarized in Table 2. These data show that in the presence of two balancer chromosomes, \textit{cup} has a dominant effect on meiotic \textit{X} chromosome segregation (Table 2) that is independent of \textit{otu}. Similar results were obtained when \textit{cup\textsuperscript{4}} was substituted for \textit{cup\textsuperscript{15}}.

\textbf{Fig. 8.} Cup affects the structure and disjunction of oocyte chromosomes. (a-f) DAPI-stained nuclei from wild-type and \textit{cup\textsuperscript{1}} oocytes, stage 5 egg chambers. (a) Oocyte nucleus from wild-type female, with chromosomes condensed into a karyosome. (b-f) \textit{cup\textsuperscript{1}} oocyte nuclei with chromosome arms radiating from DAPI-bright center. (g) DAPI image of blastoderm embryo, progeny of \textit{cup\textsuperscript{1}} female, with abnormally condensed nuclei; (h) same as g, 100× magnification.

Because \textit{cup} affected the segregation of the germ-line chromosomes, we examined the appearance of the oocyte nucleus during oogenesis for any abnormalities. In wild-type females the oocyte arrests in meiotic prophase and condenses into a small karyosome during stage 3 (Fig. 8a). In contrast, females homozygous for strong or intermediate \textit{cup} alleles produce eggs with abnormal karyosomes. Instead of condensing completely, the chromosomes appear to be organized about a central chromocenter (Fig. 8b-f). Often, five chromosome arms that appear to be double radiate outward from the central DAPI-bright center. Sometimes the chromatin separates into two or more groups (Fig. 8e and data not shown). These observations indicate that \textit{cup} is required for the proper structure of all the germ-line chromosomes and its action is not limited to cells with polytene chromosomes.

\textbf{Maternally provided \textit{cup} is essential for embryonic development}

Immunohistochemical staining demonstrated that \textit{cup} protein is strongly expressed in stage 10 and that high levels are present in preblastoderm embryos (Fig. 6g), suggesting that maternally
Table 2. X chromosome non-disjunction in cup females

<table>
<thead>
<tr>
<th>Line</th>
<th>Maternal genotype (crossed to FM6 males)</th>
<th>X chromosome genotype of progeny</th>
<th>Females</th>
<th>Males</th>
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Females homozygous for cup13 or cup4 were tested for X chromosome disjunction by crossing to FM6 or y males, and scoring the number of expected, matroclinous and patroclinous progeny. FM6 and CyO are multiply rearranged balancer chromosomes, while y, y+, Sco and the chromosomes carrying the cup and otu alleles are unarranged. Presumptive genotypes of progeny classes are presented as deduced from visible markers; patroclinous male progeny were confirmed to be sterile. All viable progeny were scored for each cross. Because of the variation in viability, data are also expressed as a percentage of the total yield (in parentheses).

To control for background genotype, all the tested females were generated from two crosses: (1), y otu11 f/FM6; cup4/CyO x y/Y; Sco/CyO or (2) y otu11 f/FM6; cup4/CyO x y/Y; Sco/CyO. Lines 1-6 were generated from cross 1, and lines 7-9 from cross 2.

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Role of cup and otu in chromosome structure

In polytene chromosomes as many as several thousand individual chromatin strands remain precisely aligned along their lengths despite continuing replication and gene activity. Many larval and adult tissues in Drosophila become polytenic as they differentiate and most appear on cytological grounds to maintain this state for the remainder of their lives. In nurse cells the precise association of individual polytene strands becomes weakened and reorganized. The exact structure of the remodeled chromosomes remains uncertain, but they are likely to retain a significant degree of intra-strand pairing (Hammond and Laird, 1985). Both cup and otu are required for nurse cell chromosome reorganization. Nurse cells mutant for otu typically develop simple polytenes chromosomes (King and Storto, 1988), while in cup mutants nurse cell chromosomes assume a variety of forms that may represent intermediates in the reorganization process. Further study of the cup pathway should make it easier to reconstruct the normal processes of chromosome remodeling and deduce the functional importance of these changes.

At least some of Cup’s actions appear to be on higher order

DISCUSSION

We have characterized a gene, fs(2)cup, that nurse cells require to maintain their normal chromatin structure, and that is needed in the oocyte for meiotic chromosome structure and segregation. The demonstration that cup interacts with otu and fs(2)B suggests that these genes participate in a pathway or process that exerts a major effect on chromosomal behavior during oogenesis. However, Cup protein, like Otu, is found in the cytoplasm so its effects on chromosome structure are likely indirect. cup and otu also affect other processes during oogenesis, such as growth of the oocyte during mid oogenesis, envelopment of the egg chamber by follicle cells, and polarization of the cyst along the anterior-posterior axis of the ovariule. It is therefore likely that the cup-otu pathway is not specific for chromosome structure and function, but is more generally required for germ line growth and development.
chromatin packing, as suggested by the specific chromosome-nuclear membrane associations observed in the cup allele. The failure of the karyosphere within the oocyte to compact fully is also evidence of altered chromatin packing. In cup germlinal vesicles, individual chromosome arms appear to be associated together in their centromeric regions, and frequently appear double. Karyosome condensation and heterochromatin-mediated association of homologous chromosomes are thought to be important for the disjunction of non-exchange chromosomes (Dernburg et al., 1996; Hawley et al., 1993). The loosening of the normal association may allow some non-exchange chromosomes to break free (Fig. 8c), leading to their failure to segregate with a partner.

**cup and otu affect oocyte growth**

In addition to disrupting chromosome organization, class III cup alleles affect the relative growth of the oocyte compared to the nurse cells during vitellogenesis. Oocyte growth depends on the influx of materials from several external sources. Nurse cells synthesize and transport components through the ring canals, while follicle cells and the fat body produce yolk proteins that are taken up at the oocyte surface. The growth defects of cup oocytes are probably not related to defects in yolk protein uptake, because mutations in the vitellogenin receptor that completely block yolk uptake do not prevent the oocyte from attaining its normal size (see Schonbaum et al., 1995). Instead, cup is more likely to interfere with transport from the nurse cells. The microtubule cytoskeleton is extensively reorganized after stage 7, and a variety of evidence suggests that microtubule-independent transport systems increase in importance during the final growth stages of oogenesis (reviewed by Theurkauf, 1994).

The large aggregates of Cup protein observed in nurse cells may play a role in oocyte transport. The transport of some specific egg components, including bicaudal (bcd) mRNA, is dependent on the gene exuperanita (exu, St Johnston et al., 1989). Exu protein is found in nurse cell cytoplasm in large aggregates that have been proposed to transport bcd mRNA along microtubules to the oocyte (Wang and Hazelrigg, 1994). Materials en route to the oocyte via this pathway may first move to the periphery of the nurse cells, because bcd RNA is seen to accumulate near nurse cell plasma membranes in an exu-dependent manner (St Johnston et al., 1989). However, Exu protein does not localize near the surface of the nurse cells in stages 9-10, and the eggs laid by exu females are not cup-shaped. Mutations in a small group of female sterile genes that includes Bicaudal-C (Mahone et al., 1995) and Bicaudal-D (Swan and Suter, 1996) more closely resemble cup’s effect on oocyte growth (Schüpbach and Wieschaus, 1991). Both genes are involved in localizing morphogenetic determinants within the oocyte. Whether cup participates in any of these pathways will require further study.

**Role of the cup-otu pathway in early germ-line development**

The cup-otu pathway is also likely to act early in oogenesis prior to the establishment of individual egg chambers. Disruption of otu activity causes germ-line cells to be lost (QUI alleles) or to overproliferate (ONC alleles), leading to tumor formation. Cup protein is expressed in forming cysts and may even be found in stem cells. The fact that cup mutations enhance tumor formation in otu suggests that both proteins function during the earliest stages of cyst differentiation. The strongest cup mutations recovered, in contrast, cause only a relatively minor increase in the number of cells in region 2a. However, none of the cup alleles tested was null, so lethality or partial redundancy may have prevented the recovery of female sterile cup alleles with stronger and earlier-acting effects.

One early process affected by cup and otu is egg chamber polarity. In the lens-shaped cysts of region 2b, the oocyte is located near the middle of the cyst but must move to a position at the posterior prior to egg chamber budding. When this process fails a dicephalic egg chamber is produced whose nurse cells are located at both ends of the egg. Signals received by the oocyte from an unidentified subpopulation of somatic cells are thought to be important for correct oocyte positioning. The fact that nearly all the egg chambers produced by cup females are dicephalic suggests that these oocytes cannot efficiently receive the polarization signal. The defects several class I alleles display in the budding of individual egg chambers may also result from a failure of signaling with somatic cells (Fig. 2a).

Previously, otu has been implicated in one the major early events of germ-line development: sex determination (Bopp et al., 1993; Pauli et al., 1993). More recent data suggest that the effects of otu on sex determination are indirect (Bae et al., 1994; Horabin et al., 1995). We found no evidence that cup functions in sex determination; ovaries from cup females showed no indication of germ-line sex transformation, and antibody staining for Sxl protein appears normal in cup mutants (L. N. K., unpublished results).

**Nature of the Cup-Otu interaction**

Cup and otu appear to function in a similar manner in organizing nurse cell chromosome structure, but some other aspects of their phenotypes differ. Both proteins are distributed in a similar manner during oogenesis, suggesting that they are located in close proximity or even in direct contact. The otu allele we analyzed, otu, contains a point mutation in the differentially spliced exon that is specific for Otu p104, an isoform that can provide complete otu function, but does not affect the other Otu isoform, Otu p98. The mutant p104 protein is temperature sensitive and retains partial function. The genetic interactions between cup and otu suggest that the relative ratios of Cup and Otu proteins, rather than their absolute levels, are important. Lowering the dose of otu increases the amount of Cup protein present in cup homzygous ovaries, suggesting that the presence of excess Otu reduces the synthesis of Cup protein or alters its stability. However, additional alleles of otu will need to be examined, including null alleles, before this can be concluded with confidence. Preliminary experiments reveal that the otu null is temperature sensitive and the enhanced tumorigenicity of the double mutants suggests that these genes may act together or in parallel.

**The cup-otu pathway may involve microtubule-based transport**

Despite the diversity of effects resulting from perturbations in cup function, many of them are known to involve microtubules.
The distribution and relocalization of Cup protein during egg chamber maturation suggest that it is directly or indirectly associated with microtubules, at least during previtellogenic stages. In early stages Cup accumulates at the oocyte posterior, as expected if its movement was associated with a minus-end directed motor (Clark et al., 1994; Theurkauf et al., 1992). Cup does not appear to accumulate in the oocyte after the dispersal of the MTOC at stage 7. Less is known about the organization of the microtubules in late-stage nurse cells; they are found concentrated around the nuclei (Gutzeit, 1986), but their polarity is uncertain. We found a weak similarity between a small domain within Cup and Map1B, while Tironnen et al. (1995) described a potential microtubule-binding domain in Otu. In addition, the proteins in the pattern database that are most closely related to Cup are involved in vesicle transport and fusion; at least one of these, CLIP-170, has been demonstrated to bind microtubules (Pierre et al., 1992). Our identification of a potential membrane-spanning domain in Cup is consistent with a role in vesicle-mediated transport.

An association with microtubules is also supported by the similar effects exerted by cup and nod on meiotic chromosome segregation. Nod encodes a kinesin-related microtubule motor protein required for the proper disjunction of non-recombining chromosomes. In the presence of multiple balancers nod, like cup, exerts a dominant effect on meiotic disjunction (Carpenter, 1973). nod has been shown to mediate the association between the centromeric heterochromatin and the microtubules of the meiotic spindle (Afshar et al., 1995; Murphy and Karpen, 1995). The ability of cup to disrupt chromosome segregation suggests that cup may also affect the ability of chromosomes to associate with the meiotic spindle.

**Association of Cup with the nuclear membrane**

Interactions between chromosomes and the nuclear membrane are likely to be important for chromosome structure. Cup is closely associated with the nuclear membrane during the stages when chromosomes are most condensed, and may indirectly influence chromatin structure by acting in a pathway that affects the nuclear membrane. Profound changes may take place in early nurse cells that are required to reorganize nurse cell chromosome structure during this period. The failure to make these changes would explain why nurse cell chromosomes remain arrested with structures characteristic of early stages. This is particularly true in the case of cup, in which the chromosomes spiral around the nurse cell nuclei in close association with the nuclear membranes (Fig. 2d).

Defective nuclear membranes could explain other effects of cup and otu mutations. For example, Otu and Cup proteins accumulated selectively in the oocyte may alter its nuclear membrane to allow it to respond appropriately to somatic signals for oocyte polarization. Still later, these proteins could act directly or via imported effectors to influence the condensation of the karyosome. Eggs in many species accumulate excess nuclear membrane constituents, including nuclear pore complexes, and store them in the cytoplasm. These materials often form at the nuclear periphery, where they assemble into large organelles known as annulate lamellae. Such stored components might correspond to the large particulate material observed in the nurse cell cytoplasm. Although annulate lamellae have been described in late Drosophila egg chambers and near the nurse cell nuclei in mid-vitellogenic stages (Mahowald, 1971), their distribution throughout oogenesis is not known with sufficient precision to compare with the location of Cup protein.

We thank Trudi Schüpbach for providing EMS-induced cup alleles, Lin Yue for molecular work on 4506, and Allison Pinder for assistance with DNA sequencing. Members of the Spradling laboratory provided much appreciated comments on the manuscript. This work was supported by grant GM27875 from the National Institutes of Health. L. N. K. was a recipient of a National Institutes of Health Postdoctoral Fellowship.

**REFERENCES**


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