The Meiotic Cell Cycle and Sister-Chromatid Cohesion in Drosophila Oocytes

by

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A.B. History and Science
Harvard and Radcliffe Colleges, 1989

Submitted to the Department of Biology
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Doctor of Philosophy in Biology

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ABSTRACT

Oocytes of many animals have highly regulated meiotic cell cycles where development and fertilization are coupled to the arrest and resumption of meiosis. This thesis investigates the meiotic cell cycle in oocytes from *Drosophila melanogaster*, whose cell cycle normally arrests at metaphase of meiosis I when the egg is mature. Two mutants, *grauzone* and *cortex*, are described which, in addition to the normal arrest in metaphase I, inappropriately arrest the meiotic cell cycle in metaphase II. Although eggs laid by mutant mothers slowly progress into anaphase II, they cannot exit the second meiotic division. To better understand the meiotic cell cycle in wild-type oocytes, an in vitro activation procedure was developed to allow observation of the stages of meiosis after metaphase I. This method is used to determine that newly translated proteins are not required for the completion of meiosis after the metaphase I arrest.

One target of meiotic regulation is sister-chromatid cohesion, which must be released appropriately to allow chromatids to segregate. The localization of the MEI-S332 protein, known to be required for sister-chromatid cohesion from anaphase I until anaphase II, is examined throughout Drosophila oocyte meiosis with the in vitro activation method. MEI-S332 localizes to the centromeric regions of meiotic chromosomes until sister chromatids separate at anaphase II. Although the protein is no longer visible at anaphase II, levels of MEI-S332 do not decrease, suggesting that the protein is not degraded at anaphase II. MEI-S332 also localizes to the centromeric regions of mitotic chromosomes before anaphase, suggesting that it may function in mitosis.

To understand the regulation and mechanism of MEI-S332-mediated sister-chromatid cohesion, interacting proteins are identified in a yeast two-hybrid screen. By this assay, the microtubule-associated kinase LK6 interacts with MEI-S332, and this interaction is confirmed in vitro. The mitotic phenotype of *mei-S332* loss-of-function mutants is strikingly similar to the overexpression phenotype of LK6, suggesting that these two proteins are in a negative regulatory relationship. The possibility that LK6 may inactivate sister-chromatid cohesion is discussed.

Thesis Supervisor: Terry L. Orr-Weaver
Title: Associate Professor of Biology

Dedicated to

my brother Justin Tuman Page (1953 - 1995)

and my grandmother Jo Wilder Abraham (1905 - 1994)

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Chapter One

Regulation of the Meiotic Cell Cycle and Sister-Chromatid Cohesion

Some of the material in this chapter has been previously published:
A. W. Page and T. L. Orr-Weaver, Stopping and Starting the Cell Cycle, Current Opinions in Genetics and Development 7, 23-31.

In eukaryotic cells, the orderly progression of growth and division is governed by the cell cycle. The mechanisms of mitotic cell cycle regulation have been remarkably conserved in widely divergent eukaryotic lineages. The meiotic cell cycle is generally viewed as a variation on the mitotic cell cycle. At the most basic level, the meiotic cell cycle requires two rounds of chromosome segregation without an intervening S-phase in order to produce haploid gametes. In higher eukaryotes, the meiotic cell cycle must be developmentally coordinated with the differentiation and growth of specialized gametes such as animal eggs and sperm. This developmental coordination requires levels of regulation in meiosis that are not required in most mitotic divisions. Additionally, although in some organisms gametes are able to survive as haploids, in many animals gametes have a short period of viability during which time they must accomplish karyogamy (pronuclear fusion) if the gametes are to survive. In these animals, the female meiotic cell cycle is coordinated with external events to ensure timely karyogamy. Thus the female meiotic cell cycle in most animals has additional levels of regulation not present in mitosis. These additional levels of cell cycle regulation are often observed as arrests and resumptions of the meiotic cell cycle.

This thesis concerns the female meiotic cell cycle in the fruitfly *Drosophila melanogaster*. The work described here is divided roughly into two parts. Chapters 2 and 3 investigate the meiotic cell cycle, largely unexplored in Drosophila, with the aim of elucidating the mechanisms governing the arrest and resumption of the meiotic cell cycle in females. The second part investigates the link between the cell cycle and chromosomal behavior, the ultimate target of meiotic regulation in oocytes. Specifically, sister-chromatid cohesion and its regulation by the meiotic cell cycle are explored in Chapters 4 and 5. During meiosis, the chromosomes must be segregated correctly at two divisions, with homologous chromosomes segregating at meiosis I and sister chromatids segregating at meiosis II. In order for both these divisions to proceed properly, cohesion between sister chromatids must be maintained and released at the correct times, as dictated by the meiotic cell cycle. The misregulation of sister-chromatid cohesion results not only in aneuploid gametes, but also in changes in the timing of the divisions.

This chapter will give an overview of the meiotic cell cycle and sister-chromatid cohesion. Key regulators of the meiotic cell cycle that have been identified in different organisms are examined first. The meiotic cell cycle of Drosophila oocytes is then introduced in detail. Next, the requirements for sister-

chromatid cohesion in meiosis and mitosis are discussed. Finally, the current understanding of mechanisms of cohesion and release of cohesion are explored.

REGULATION OF THE MEIOTIC CELL CYCLE

Many key regulators of the meiotic cell cycle have been identified in oocytes of animals such as frogs, starfish, and sea urchins, animals that lay many eggs at once and so are useful for biochemical studies. Animal oocytes arrest during a stage similar to G2 that is actually meiotic prophase I, as the oocyte grows and differentiates. At the initiation of maturation, developmental signals induce prophase I-arrested oocytes to resume the meiotic cell cycle. In many species, a mature oocyte that has been released from prophase I stops again at a second developmental arrest, usually awaiting fertilization (for a very good review, see Sagata, 1996). These second arrest points vary greatly in different organisms and are listed in Fig. 1-1. Molecular regulation of the meiotic cell cycle is best understood in Xenopus oocytes, and I will present here a summary of the major events.

Resuming the Meiotic Cell Cycle after the Prophase I Arrest

In Xenopus, progesterone stimulates fully developed prophase I-arrested oocytes to re-enter the cell cycle. Cytologically, cell-cycle resumption is evident by chromatin condensation, germinal vesicle breakdown (GVBD), and extrusion of the first polar body. On a molecular level, progesterone stimulation increases the activity of two important regulatory kinases that are not specific for meiosis: maturation promoting factor (MPF or cdc2/cyclin B) and the mitogen activated protein kinase (MAPK). Cdc2 is a universal regulator of the cell cycle, and MAPK is used in many cell types in all eukaryotes to transduce signals. The meiosis specific serine/threonine kinase mos is responsible for many of the unusual characteristics of the meiotic cell cycle in Xenopus oocytes. Mos is required for re-initiation of the cell cycle after the prophase I arrest, for the inhibition of DNA synthesis between the meiotic divisions, and for the second arrest at metaphase II (for reviews see Gebauer and Richter, 1997; Sagata, 1997).

For a long time it has been known that translation of new protein(s) was required for release from the prophase I arrest in Xenopus oocytes (Gerhart et al., 1984; Wasserman and Masui, 1975). It is now clear that mos, a proto-oncogene, must be translated for maturation to proceed. Ablation of the mos message inhibits progesterone-stimulated GVBD (Sagata et al., 1988). The translation of

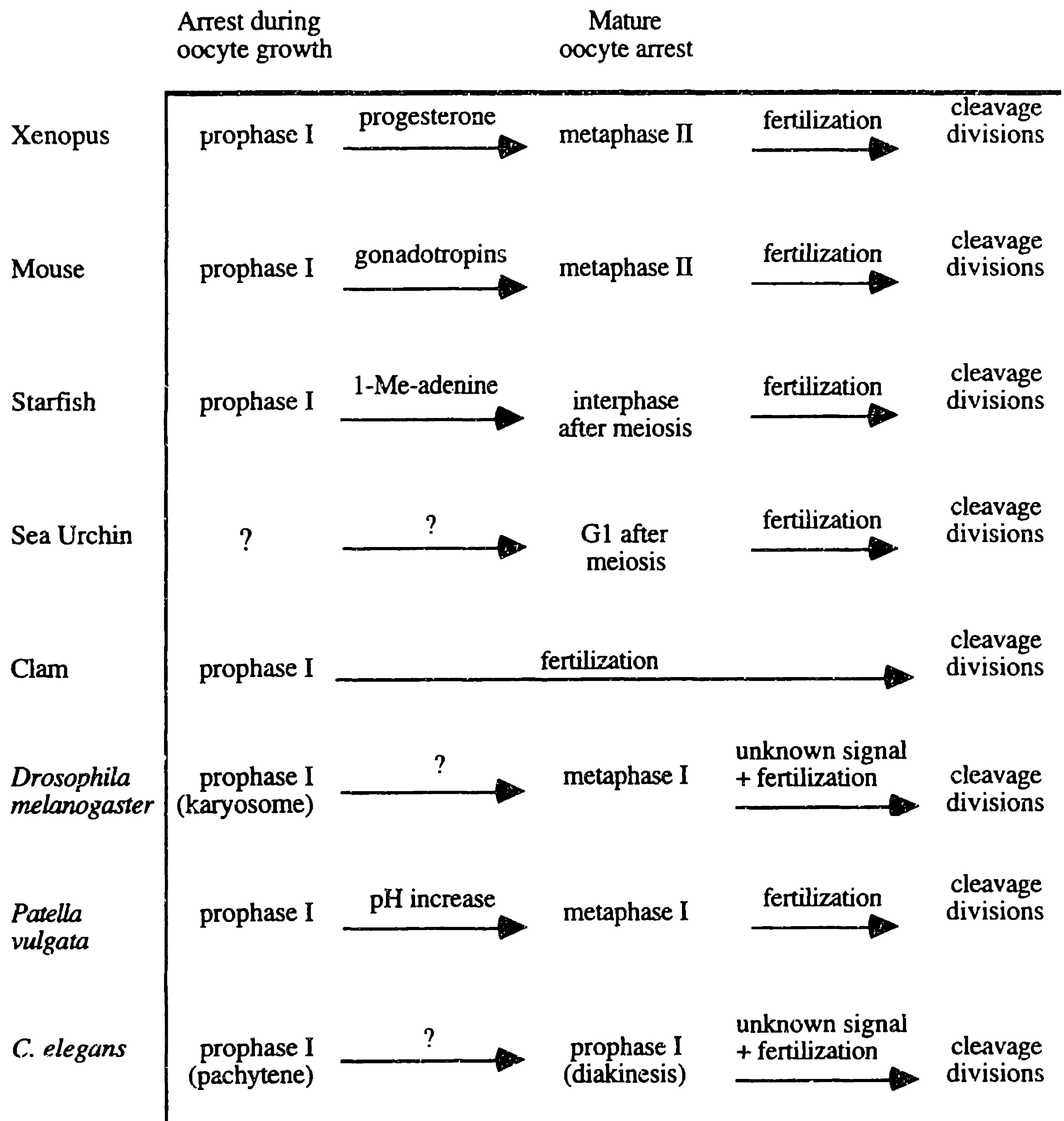


Figure 1-1. Meiotic arrest points of different animal oocytes.

Oocytes of most species arrest twice in meiosis, first at prophase I and then at another point that varies depending on the organism. Only some of the signals releasing these arrests are known. The question marks indicate an unknown signal.

mos is regulated in part by polyadenylation of the mos message, and amputation of its polyA tail also inhibits GVBD (Sheets et al., 1995). Conversely, injection of high concentrations of mos protein can trigger GVBD in the absence of progesterone (Yew et al., 1992).

It has been demonstrated *in vivo* and *in vitro* that mos activates MAPK, probably by phosphorylating MAPK kinase (MAPKK; Nebreda et al., 1993; Nebreda and Hunt, 1993; Posada et al., 1993; Roy et al., 1996; Shibuya and Ruderman, 1993). Maturation can be achieved by injecting MAPK protein or mRNA encoding constitutively active MAPKK, but this maturation also requires protein synthesis (Gotoh et al., 1995; Haccard et al., 1995; Roy et al., 1996). These experiments and others have established the model that at maturation, progesterone stimulates Xenopus oocytes to translate mos; mos activates the MAPK pathway and other targets; and MAPK is indirectly responsible for converting a preformed pool of inactive MPF into active MPF (figure 1-2). *In vitro* experiments have supported this model, although they do not reproduce the *in vivo* events exactly (Huang and Ferrell, 1996).

The precise chain of events from progesterone stimulation to mos polyadenylation and translation are not clear. However, once mos translation is initiated, the mos protein is initially unstable (Nishizawa et al., 1992). It appears that there is a feedback loop between mos, MAPK, and MPF that acts to stabilize mos and allow GVBD to proceed (Gotoh et al., 1995; Nebreda et al., 1995). Injections of dominant-negative $p34^{cdc2}$ into oocytes inhibited progesterone-induced GVBD by inhibiting the accumulation (but not the translation) of mos protein. In these oocytes, there was not sufficient accumulation of mos to activate the MAPK pathway (Nebreda et al., 1995). Thus cdc2 activity may be required for the stabilization of mos protein and the resulting activation of the MAPK pathway, which in turn is required for cdc2 activation. How such a feedback loop is initiated is still unclear.

Inhibiting Replication between the Meiotic Divisions

Once a Xenopus oocyte has resumed the cell cycle at maturation, the oocyte completes meiosis I and proceeds to meiosis II. The inhibition of protein synthesis blocks this transition into meiosis II, demonstrating that new proteins are required to enter meiosis II (Gerhart et al., 1984; Yew et al., 1992). As at GVBD, new translation of mos is required for progression into meiosis II (Kanki and Donoghue, 1991). One of the most intriguing aspects of meiosis is that between

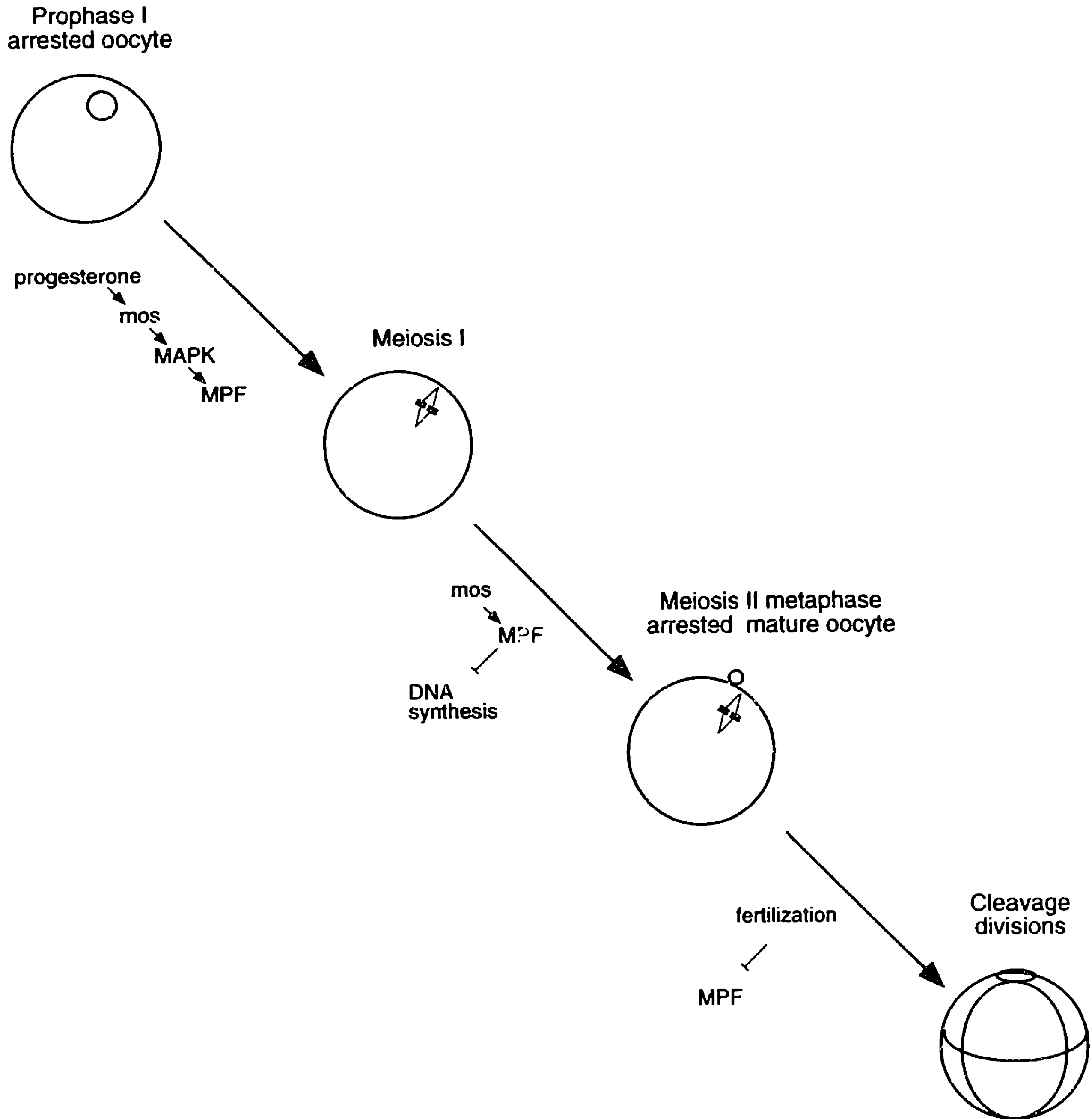


Figure 1-2. Xenopus oocyte maturation.

Progeterone stimulates the release of the prophase I arrest, acting through mos and MAPK to activate MPF and resume meiosis. MPF and mos are also required to repress DNA synthesis in the transition between meiosis I and the metaphase II arrest.

these divisions the nucleus does not undergo DNA replication. Although originally it was believed that MPF levels dropped between the two meiotic divisions, recent studies of H1 kinase activity (a measure of all cdc2 activities) show that activity drops prematurely at metaphase I and then rises during anaphase I so that between the meiotic divisions the H1 kinase activity is high (Furuno et al., 1994; Ohsumi et al., 1994). This high H1 kinase level may be required to inhibit S-phase. In support of such a model, the injection of dominant-negative cdc2 into both Xenopus and starfish oocytes allows replication between the divisions (Furuno et al., 1994; Picard et al., 1996).

Mouse oocytes, which do not require either mos or protein synthesis to initiate GVBD, do display a drop in MPF activity between the meiotic divisions (Choi et al., 1991; Hashimoto and Kishimoto, 1988). However MAP kinase activity remains high and is probably responsible for inhibiting interphase from occurring between the divisions. As in oocytes of most species, chromatin in wild-type mouse oocytes remains condensed between the meiotic divisions, and this continuing chromatin condensation may be important for inhibiting replication between MI and MII. In wild-type oocytes, the extent of chromatin condensation throughout meiosis is correlated with the levels of MAPK (Verlhac et al., 1994). Moreover, in *mos-/-* oocytes, which cannot activate MAPK, the chromatin becomes slightly decondensed between the two divisions (Choi et al., 1996; Verlhac et al., 1996). Thus MAPK appears to be required for maintenance of chromatin condensation between MI and MII. It would be interesting to know if mouse *mos -/-* oocytes are capable of replication between MI and MII, since it has been demonstrated that the ablation of mos message allows replication between the divisions in Xenopus oocytes (Furuno et al., 1994).

Mos, perhaps acting through the MAPK pathway, is also required for the meiosis-specific morphology of the spindle (Choi et al., 1996; Verlhac et al., 1996). Defects in the spindles of *mos -/-* oocytes include misorientation of the spindle with respect to the oocyte surface, and the formation of an abnormally large polar body (Choi et al., 1996). This phenotype can be interpreted as the imposition of mitotic control during meiosis I, suggesting that mos supplies meiosis-specific functions. In support of this interpretation, the overexpression of mos in mammalian mitotic 3T3 cells causes a number of meiotic-like transformations. The spindles become anastral, typical of meiotic spindles in mice and many other organisms, and the spindles attach to one side of the outer membrane as a meiotic spindle does (Fukasawa and Vande Woude, 1995).

Arresting at Metaphase II

In Xenopus, mouse, and many other vertebrates, the meiotic cell cycle is arrested a second time at metaphase II. An activity known as cytostatic factor (CSF) is responsible (Masui and Markert, 1971), and the only component of CSF so far identified is mos (Sagata et al., 1989). Mos was identified as a likely CSF component based on immunodepletion of extracts and its ability to impose a CSF-like arrest when injected into cleavage-stage embryos (Sagata et al., 1989). Genetic evidence from mouse knock-out studies confirms the functional importance of mos in CSF activity. In contrast to wild-type mouse oocytes which arrest at metaphase II in response to CSF, oocytes from *mos -/-* mice do not arrest at metaphase II (Colledge et al., 1994; Hashimoto et al., 1994).

Extracts from metaphase II arrested oocytes have demonstrated that cyclin B is stable as long as the arrest is maintained, and this keeps MPF activity high (Murray et al., 1989). Resumption of the cell cycle requires the ubiquitin-mediated degradation of cyclin B (Glotzer et al., 1991); the complex responsible for targeting cyclin B for degradation is called the anaphase-promoting complex (or APC; King et al., 1995). Originally CSF was believed to stabilize MPF by inhibiting cyclin B proteolysis (Murray et al., 1989), but CSF may not block APC activity directly as new evidence suggests the APC may be active during the metaphase II arrest. APC activity is highly correlated with the mobility of its Cdc27 subunit in mitosis (King et al., 1995); the mobility of Cdc27 in meiosis suggests that the APC may be active throughout the meiotic cell cycle from the induction of GVBD up to and including the metaphase II arrest (Thibier et al., 1997). Interestingly, it has been demonstrated that there are two separate pools of cyclin B during that arrest, one sensitive to cycloheximide and one stable in cycloheximide (Thibier et al., 1997). It is likely that the unstable pool is being degraded by the APC and replenished with new synthesis, but the stable pool is somehow protected by CSF. The question of how the CSF arrest is maintained in the presence of an active APC has yet to be resolved.

The metaphase II arrest is released at fertilization. Fertilization causes a transient increase in calcium levels, and this indirectly leads to the inactivation of MPF and CSF. MPF is inactivated by calcium-stimulated degradation of its cyclin B subunit (Murray and Kirschner, 1989); it has been assumed that a calcium-dependent protease degrades mos, thus inactivating MPF (Lorca et al., 1993; Watanabe et al., 1989). The loss of MPF activity precedes the loss of CSF

activity (Watanabe et al., 1991), demonstrating that after fertilization CSF loses its ability to protect cyclin B from destruction. The inactivation of CSF is required by the first embryonic mitosis so that it does not arrest inappropriately at metaphase.

THE MEIOTIC CELL CYCLE OF *DROSOPHILA MELANOGASTER*

An analysis of the meiotic cell cycle in an organism amenable to genetic dissection could be an important complement to the molecular and biochemical studies in Xenopus oocytes. *Drosophila melanogaster* is such organism, with a long-standing tradition of powerful genetic analysis. Mutant hunts could identify meiosis-specific functions in oocytes and mutant analysis could lead to new insights. Additionally, studies in the meiotic cell cycle of diverse organisms will lead to an appreciation of conserved molecular and biological functions. Here I present an overview of the events in Drosophila oocyte meiosis, and what is known about the mechanisms underlying these events.

Drosophila oogenesis begins in the germarium, the tissue that houses the germ-line stem cells. A single cystoblast divides four times to form 16 cells with interconnected cytoplasm; this is an egg chamber. Of these 16 cells, one will develop as the oocyte nucleus, and the other 15 will become nurse cells that nourish the oocyte. Pre-meiotic S phase begins immediately after the divisions that create the egg chamber. It has been difficult to stage events within meiotic prophase because the chromosomes are so tightly compacted that the cytology is very poor, but it is believed that prophase is initiated inside the germarium because synaptonemal complex is visualized there (Carpenter, 1975). Egg chamber development has been divided into 14 stages based on morphological criteria (King, 1970; Mahowald and Kambysellis, 1980) and from stages 3 - 13, the oocyte nucleus takes on a distinctive tightly packed morphology called the karyosome. Thus the first meiotic arrest in Drosophila oocytes is at the karyosome stage of meiotic prophase, and this arrest lasts for about two days (for reviews of oogenesis, see Mahowald and Kambysellis, 1980; Spradling, 1993).

At stage 13, the karyosome decondenses, the nuclear envelope breaks down, and assembly of the meiosis I spindle begins (Mahowald and Kambysellis, 1980; Theurkauf and Hawley, 1992). There is no evidence that centrosomes are present in the Drosophila oocyte during meiosis (Matthies et al., 1996; Theurkauf and Hawley, 1992), but it has been established that the minus-end directed motor NCD is required to bundle microtubules at the spindle poles and maintain

spindle stability (Matthies et al., 1996). The centrosomal component gamma-tubulin is also required for proper spindle morphology (Tavosanis et al., 1997). It is likely that the chromosomes themselves play an important role in organizing the meiotic spindle because in mutants where chromosomes become separated from the meiosis I spindle, mini-spindles assemble around the lost chromosomes (Theurkauf and Hawley, 1992). By stage 14, the last stage of oogenesis, the meiosis I spindle is assembled and the oocyte arrests at metaphase I.

Generally, in meiosis I each pair of homologs, called a bivalent, is pulled towards opposite poles by kinetochore spindle attachments. In spite of this pole-ward force, metaphase I is maintained because the homologs in a bivalent are attached to each other at chiasmata, the sites of reciprocal recombination (discussed below). Direct micromanipulations in grasshopper meiosis I spermatocytes have demonstrated that the attachment of a bivalent to the spindle is only stabilized when the attachment is bipolar, that is, when each homolog of the bivalent is attached to spindle fibers from opposite poles (Nicklas, 1967). When bipolar attachments are not achieved, the bivalent reorients and attempts again to attain bipolar attachment. Experimentally, however, attachments to the same pole can be stabilized if a microneedle pulls the bivalent toward the opposite pole (Nicklas and Koch, 1969). These experiments have led to the generally accepted model that tension across a bivalent, or across sister-chromatids in mitosis or meiosis II, is required to stabilize the attachment between a kinetochore and a spindle fiber. When a bivalent or chromosome is maloriented, anaphase is inhibited until bipolar orientations are established for all the chromosomes; experimentally applied tension to a maloriented bivalent allows the cell to proceed into anaphase under conditions when it would normally be inhibited (Li and Nicklas, 1995).

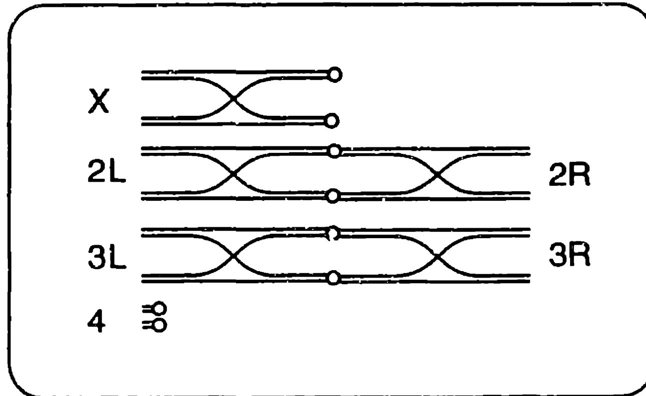
In Drosophila, meiotic mutant oocytes that have no recombination events, and thus no chiasmata to hold bivalents together, do not arrest at metaphase I as wild-type oocytes do (McKim et al., 1993). From the experiments described above, it might be expected that bivalents lacking chiasmata might prohibit anaphase from proceeding; however the opposite appears to be the case. Perhaps even more interesting is that although it might be expected that the chromosomes unable to form a metaphase I plate would arrest instead at anaphase or telophase I, it appears that chromosomes in these oocytes proceed into meiosis II. Yet if only one set of homologs has recombined, this one bivalent is sufficient to allow the oocyte to arrest at metaphase I (McKim et al., 1993).

Two hypotheses have been proposed to explain why a single set of recombinant homologs is required for the metaphase arrest: either something about recombination itself is required, or else a bipolar attachment of a bivalent to the spindle is required to oppose the poleward forces. To distinguish between these hypotheses, Jang et al. constructed females that carried only compound chromosomes, in which each pair of homolog arms was attached to the same kinetochore (Figure 1-3) (Jang et al., 1995). In oocytes from such females, recombination can proceed but it does not result in a chiasma attaching two kinetochores; thus homolog pairs are unable to make bipolar attachments to the spindle. In oocytes from these all-compound females, there was no metaphase I arrest. Thus, the presence of one set of homologs with bipolar spindle attachments (and not recombination) allows the cell to maintain a metaphase I configuration for all the chromosomes.

The metaphase-I arrest is maintained in the mature oocyte until the oocyte is activated, up to several days later. Activation occurs as the oocyte moves from the ovary into the uterus via the oviduct, which is lined with fluid that is believed to activate the oocyte (Mahowald et al., 1983). At activation, the oocyte may properly be called an egg. Once in the uterus, the newly activated egg is usually fertilized by sperm stored in the seminal receptacle and spermatothecae (Miller, 1950). Thus, release from the second meiotic arrest and fertilization are coupled under normal circumstances. If however there are no sperm, unfertilized eggs do become activated and complete meiosis (Rabinowitz, 1941). Whether or not fertilization has been accomplished, after activation meiosis I is completed rapidly, meiosis II follows immediately, and the meiotic divisions are often complete by the time the egg is laid, within about 20 minutes. Recent studies on live oocytes visualizing NCD, which localizes to meiotic spindles, has shown that the meiosis I spindle does not depolymerize between the divisions, but simply elongates into the two meiosis II spindles (Endow and Komma, 1997).

All four products of the Drosphila oocyte meiotic divisions are held within the egg, rather than extruded as in vertebrates. At the end of meiosis these meiotic products decondense. If the egg is fertilized, the male pronucleus also decondenses, and the male and female pronuclei migrate together into the center of the embryo, where they undertake the first mitotic division. The other three meiotic products cluster together in the dorsal anterior quadrant of the embryo and condense their chromosomes into morphologically distinct polar body

A Exchange events in a wild-type Drosophila oocyte



B Exchange events in an all-compound Drosophila oocyte

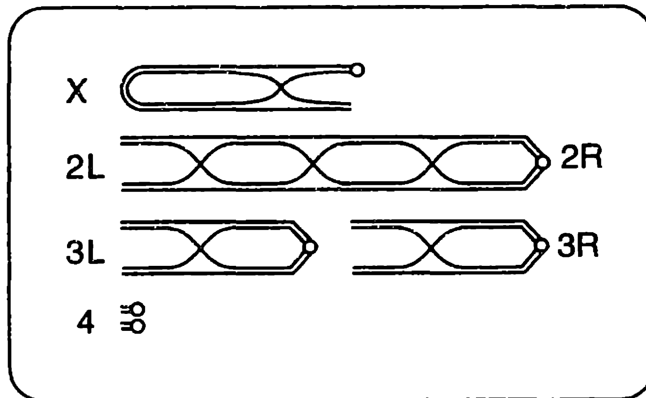


Figure 1-3. Cross-overs between compound chromosomes do not allow bipolar spindle attachment.

Exchange events (meiotic crossing-over) in a normal oocyte (A) connect homologous kinetochores, allowing bipolar attachment of the bivalent to the spindle. In contrast, exchange events in the all-compound oocyte (B) can still occur but exchanges do not connect homologous kinetochores.

rosettes. If the egg is unfertilized, all four meiotic products become part of the polar body rosette (for review, see Foe et al., 1993).

Surprisingly few mutations have been characterized that affect the meiotic cell cycle in Drosophila oocytes, even though dozens of mutants are known to affect recombination and meiotic chromosome transmission (Baker et al., 1976). It is plausible that this dearth of mutants reflects an underlying reality that not very many meiosis-specific cell-cycle genes exist; indeed in the better characterized Xenopus oocyte system, only mos appears to be a meiosis-specific function. However, the lack of mutant analysis is exacerbated by the fact that until recently it was difficult to observe meiosis in oocytes. A relatively new technique allows mass isolation of metaphase-I arrested oocytes, and this has enabled studies of meiosis in mature oocytes (Theurkauf and Hawley, 1992). However, the analysis of mutants after the metaphase I arrest has continued to be extremely difficult. Three factors contribute to this difficulty: the divisions happen rapidly; they take place inside the mother; and because each egg enters meiosis individually, it is not possible to recover a group of synchronized eggs undergoing meiosis.

Before the study presented in Chapter 2, the only Drosophila mutant known to affect the oocyte meiotic cell cycle was *twine* (Courtot et al., 1992; White-Cooper et al., 1993). *twine* encodes a homolog of the cdc2-activating phosphatase cdc25. Cdc2, the kinase subunit of MPF, is inactivated by phosphorylation on Tyr15; cdc25 removes the phosphate at Tyr15 and thus activates cdc2 (Solomon, 1993). Drosophila has two homologs of cdc25, *string*, required for embryogenesis (Edgar and O'Farrell, 1989), and *twine*, required for meiosis. In mature oocytes from *twine* mutants, grossly abnormal morphology was observed in place of the metaphase I arrest: spindles were asymmetrical and not bipolar; chromosomes were scattered and sometimes removed from the spindle; and sometimes more than one spindle was observed (White-Cooper et al., 1993; the observations of Courtot et al. [1992] must be viewed with caution since they were made under conditions that activate oocytes). Although these observations were interpreted as a failure of the metaphase I arrest and a continuation of meiosis, the cytology did not resemble the orderly progression of meiosis after activation. Another interpretation is that *twine* is required for the proper transition from the prophase-I arrest into metaphase I. This interpretation is consistent with the *twine* mutant phenotype in male meiosis. In mutant males, meiosis was initiated as observed by nuclear envelope breakdown and chromosome condensation, but meiosis could not progress and no spindles were formed (White-Cooper et al.,

1993). Thus it is possible that in both sexes, meiosis is initiated but in the absence of TWINE cannot progress through meiosis I. Assuming that TWINE functions through cdc2 as its homologs do, this interpretation implies that activated cdc2 is required for the meiotic divisions. It is not required however for all aspects of meiotic initiation, such as chromosome condensation.

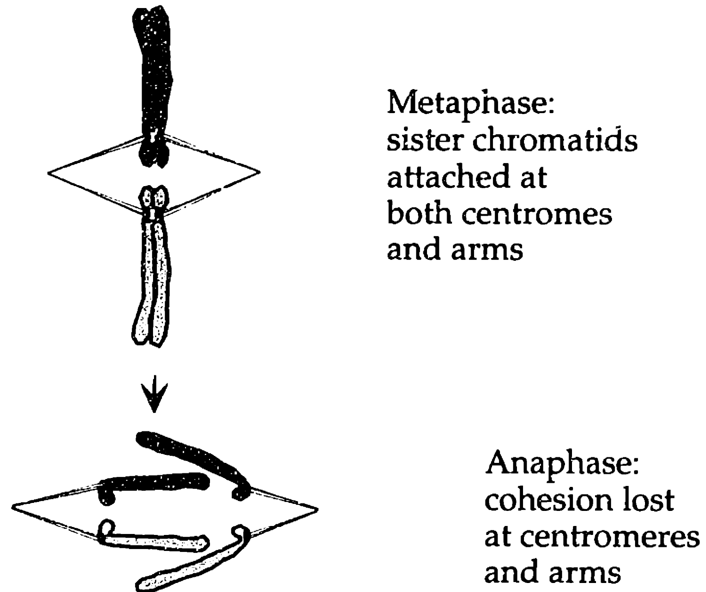
SISTER-CHROMATID COHESION IN THE MEIOTIC CELL CYCLE

In all oocytes regulators of the meiotic cell cycle must act on the downstream targets that perform meiosis-specific functions. The meiotic chromosomes are critical targets of meiotic regulation, as they must segregate correctly through two divisions. Indeed, in Drosophila oocytes, which do not undergo cytokinesis during meiosis, it would be fair to say that chromosomes are the most important targets of meiotic regulation. In meiosis of all organisms in order for the chromosomes to segregate properly, they must maintain their association with each other so that they can be properly attached to opposite spindle poles. Regulated sister-chromatid cohesion is essential for correct chromosome segregation in meiosis I and II.

Sister Chromatid Cohesion and Meiotic Chromosome Segregation

Sister chromatids lose cohesion with each other in two steps in meiosis (Figure 1-4). At the metaphase/anaphase I transition cohesion is lost along the length of the arms (John, 1990; Suja et al., 1992). At the metaphase/anaphase II transition the cohesion remaining at the centromeric regions is lost, allowing the sister chromatids to segregate away from each other (John, 1990). This two-step loss of sister-chromatid cohesion in meiosis can be contrasted to its loss in mitosis (Figure 1-4). Mitotic chromosomes are associated at both arm and centromeric regions at metaphase, and cohesion is lost all at once in both regions as the chromatids separate in anaphase.

This step-wise regulation of sister-chromatid cohesion is required for chromosomes to segregate faithfully through both meiotic divisions. In meiosis I, bivalents must attach to the spindle such that each homolog will segregate to opposite poles. Bipolar attachment can only be ensured if the homologs are first attached to each other. In meiosis of most organisms, including female Drosophila, the homologs are attached to each other as a by-product of meiotic crossing over, also called recombination. The point of attachment between two



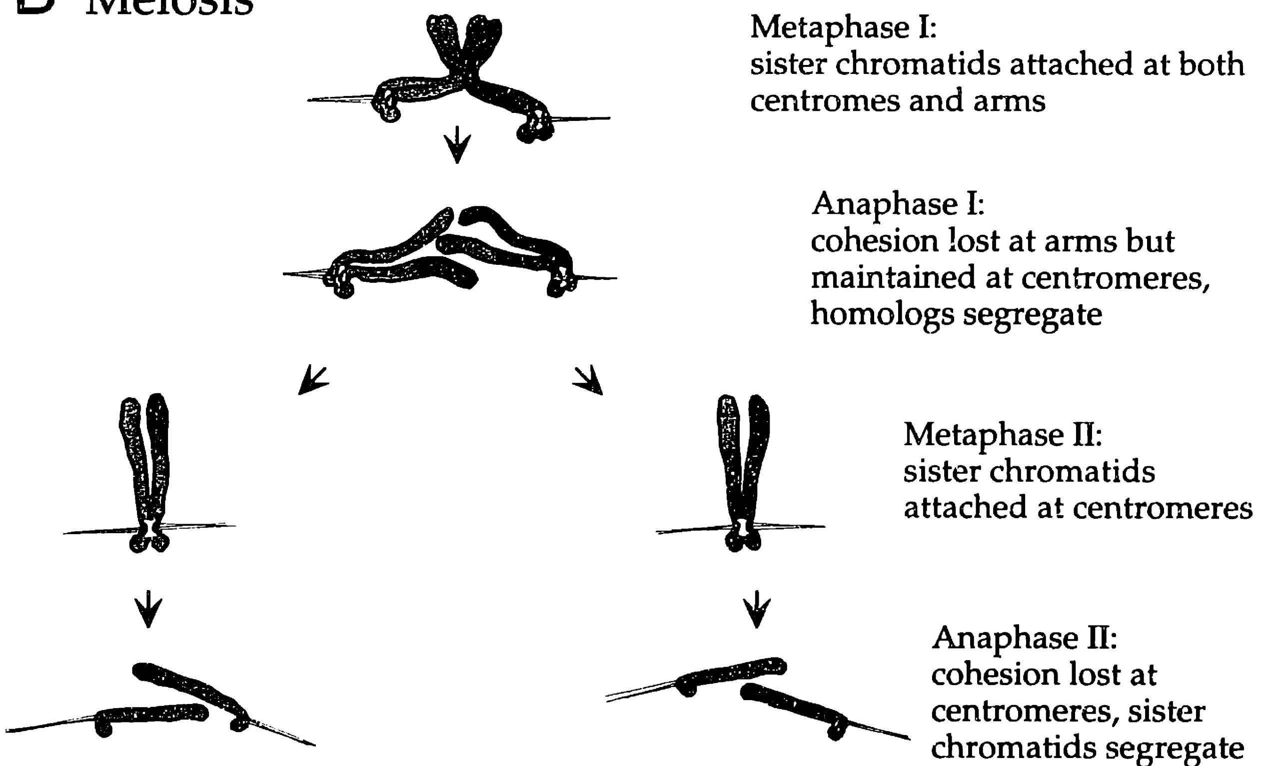


Figure 1-4. Sister-chromatid cohesion in meiosis and mitosis. Homologous chromosomes are different shades of grey; sister chromatids are the same color.
A. In mitosis, sister-chromatid cohesion is lost from the arms and centromeres at the metaphase/anaphase transition.
B. In meiosis, cohesion is lost in two steps: from the arms at the metaphase/ anaphase I transition, and from the centromeres at the metaphase/ anaphase II transition.

homolog arms is called the chiasma (plural chiasmata). Yet a chiasma is inherently unstable: it is simply one chromatid lying on top of another chromatid. Figure 1-5A illustrates how a chiasma itself is not sufficient to hold homologs together, as recombined homologs can still slide apart easily. If the paired homologs separate, they will no longer be capable of maintaining a bipolar attachment stabilized by tension. Thus, if they separate too early, each homolog will likely be detached from the spindle microtubules and then reattached to microtubules emanating from either pole. The segregation of one homolog to each pole can no longer be assured.

It is believed that a chiasma is stabilized by cohesion between the sister-chromatid arms, and this stabilization allows the homologs to remain associated (see Figure 1-5B). With arm cohesion, the homologs can remain attached even under bipolar tension from the spindle. Indeed, anaphase I cannot commence until sister-chromatid cohesion is lost along the sister-chromatid arms at the start of anaphase I. If all cohesion between sister-chromatids were lost at anaphase I, however, the sister-chromatids would not remain associated. Unassociated sister-chromatids in anaphase I could not make bipolar attachments to the spindle during meiosis II, and would missegregate at anaphase II. Thus during anaphase I and metaphase II the sister chromatids must still remain attached to each other, and this attachment is maintained at the centromeric region of sister chromatids. In sum, sister-chromatid arm cohesion allows proper orientation of homologous chromosomes on the meiosis I spindle, and sister-chromatid centromeric cohesion allows proper orientation of sister chromatids on the meiosis II spindle.

This model of different sister-chromatid cohesion requirements for each meiotic division is supported by genetic and cytological studies. One study in maize tested three models of chiasma stabilization to see whether they could predict the chromosome bridges and fragments formed at the anaphase I segregation of a heterozygous paracentric inversion. The observed chromosome configurations were best explained by the model of chiasma stabilization through sister-chromatid arm cohesion (Maguire, 1982). Single-gene mutants from many organisms have defects in sister-chromatid cohesion and chromosome segregation. In a study of maize plants mutant for *desynaptic (dy)*, recombination occurred at wild-type levels during meiosis as assessed by cytology (Maguire, 1978). After recombination however, chiasmata were not maintained, the

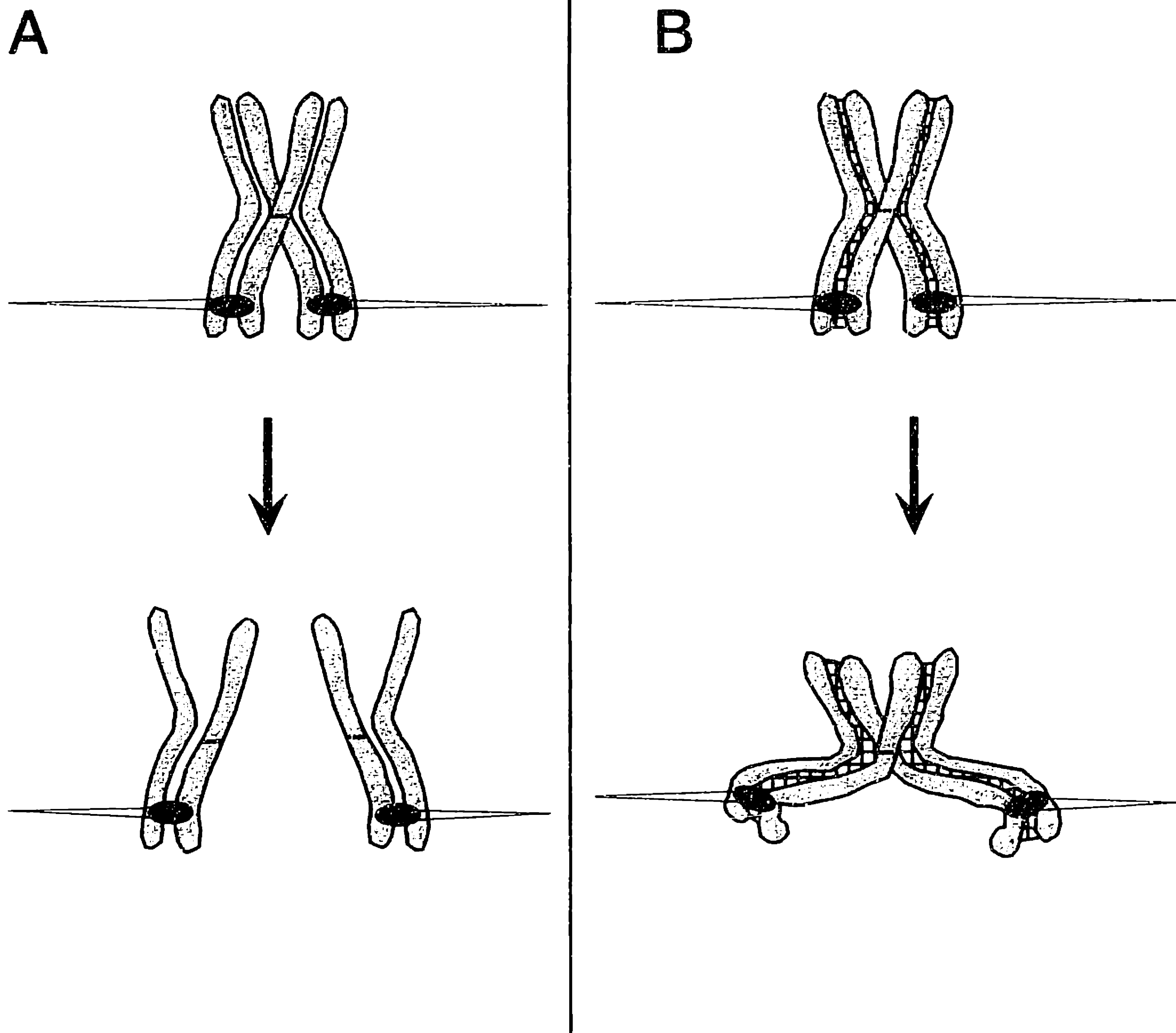


Figure 1-5. Sister-chromatid cohesion can stabilize homologous chromosomes at metaphase I by maintaining chiasmata.

A) Recombined homologous chromosomes are shown at metaphase I. The chiasma (the cross-over point) is not inherently stable; without chiasma stablization, the homologs will prematurely separate under tension from the spindle.

B) The presence of sister-chromatid cohesion (shown as cross-hatching) between the arms of the recombined chromosomes acts to stabilize the chiasma. The homologous chromosomes remain attached to each other even under tension from the spindle until sister-chromatid cohesion is dissolved along the chromosome arms at the start of anaphase I.

homologous chromosomes separated precociously, and homologs did not segregate faithfully at meiosis I. After meiosis I in *dy* mutants, the sister chromatids separated precociously in prophase II. Both the lack of association of homologs at meiosis I and of sister chromatids at meiosis II can be explained by a failure of sister-chromatid cohesion in *dy* mutants (Maguire, 1978). *REC8* is a meiosis-specific gene required for sister-chromatid cohesion in *S. pombe;* in *rec8-110* mutants, the sister chromatids segregate improperly at both divisions (Lin et al., 1992; Molnar et al., 1995). This mutant demonstrates again the requirement for sister-chromatid cohesion in the meiotic divisions.

In Drosophila, *ord* mutants are defective in sister-chromatid cohesion and segregation at both meiotic divisions. Genetic tests have demonstrated that *ord* is required in both males and females for faithful chromosome segregation in meiosis I and II, and recombination does not ensure faithful segregation (Miyazaki and Orr-Weaver, 1992). In *ord* mutant males, premature separation of sister chromatids has been observed cytologically as early as prophase I (Miyazaki and Orr-Weaver, 1992). These studies strongly suggest that *ord* is required for sister-chromatid cohesion in both sexes. Analysis of weak alleles suggests that ord is required for both arm and centromeric cohesion (Bickel et al., 1997). The cytology of *ord* oocytes shows interesting links between sister-chromatid cohesion and the oocyte meiotic cell cycle. In *ord* mutant oocytes, metaphase I does not arrest as it does in wild-type Drosophila oocytes. Like the recombination-defective mutants described above, oocytes from *ord* mothers completed both divisions without egg activation (S. Bickel and T. L. Orr-Weaver, unpublished data). This phenotype highlights the importance of sister-chromatid cohesion in controlling the oocyte meiotic cell cycle.

Drosophila mutations exist that separately affect centromeric cohesion and arm cohesion in meiosis. In *ord* mutants, the sister chromatids have no cohesion, as demonstrated by cytological and genetic data. In *mei-S332* mutants, however, the sister-chromatids remain associated until anaphase I, when they separate prematurely (Kerrebrock et al., 1992). Because the sister chromatids are no longer associated, they fail to form metaphase plates in meiosis II and cannot segregate correctly. This phenotype has been assessed cytologically in males, and genetically in both sexes by following the segregation of marked chromosomes (Kerrebrock et al., 1992). Chromosome missegregation appears to be specific to meiosis and not to occur in mitotic divisions in *mei-S332* mutants; the existence of the *ord* and *mei-S332* phenotypes suggest that meiosis-specific functions are

required to maintain sister-chromatid cohesion both along the arms and at the centromeres.

Mechanisms of Sister-Chromatid Cohesion

Much of what is known about the molecular basis for sister chromatid cohesion comes from studies of mitotic cells. From these studies, it appears that the molecular basis for sister-chromatid cohesion may be complex. It appears that sister-chromatids can be held together by the catenation of sister-chromatid DNA and/or by proteins that provide a cohesion function. Catenation of sister chromatids is a by-product of DNA replication in the preceding S-phase (Holm, 1994; Murray and Szostak, 1985), and its postulated role in chromatid cohesion comes from data demonstrating that topoisomerase II (topo II) is required for chromatids to separate at anaphase. Analysis of topo II mutants in both *S. cerevisiae* and *S. pombe* has shown that topo II is required during mitosis to allow chromatid separation (Holm et al., 1985; Uemura et al., 1987); in *S. cerevisiae* topo II mutants mitotic chromosomes nondisjoin (Holm et al., 1989). In meiosis of *S. cerevisiae,* topo II is required for progression through both meiotic divisions; the meiosis I requirement for topo II is relieved if there is no meiotic recombination, but then aberrant chromosome partitioning occurs in meiosis II (Rose et al., 1990). In Xenopus mitotic extracts, topo II inhibitors added immediately before anaphase inhibit chromatid separation at anaphase (Shamu and Murray, 1992). These data support a model in which topo II decatenates mitotic chromosomes at the start of anaphase. According to this model, in meiosis topo II is restricted to disentangling chromatid arms at anaphase I and later disentangles the centromeres at anaphase II.

Clearly topo II is required for sister-chromatid separation in mitosis and meiosis, suggesting that decatenation is required to untangle chromosomes. Yet it is clear that catenation is not the only basis for sister-chromatid cohesion because circular minichromosomes, which are not catenated (Koshland and Hartwell, 1987), maintain their association with each other in *S. cerevisiae* until anaphase (Guacci et al., 1994). This association without catenation suggests that proteins can also act as a sister-chromatid cohesive force, perhaps acting as a glue to hold sisters together. In fact, it has been demonstrated that an exogenous protein that can bind two sister chromatids together is sufficient for prolonging sister-chromatid association under conditions when they normally separate in *S. cerevisiae* (Straight et al., 1996). Although both these studies were performed on

mitotic cells, it is likely that proteins contribute to maintaining sister-chromatid cohesion in meiosis as well. If proteins hold sister chromatids together, they must either be removed or degraded at the metaphase/anaphase transition, since there is evidence that the forces generated by the mitotic spindle do not increase appreciably at the initiation of anaphase (Alexander and Rieder, 1991; Nicklas, 1988).

Of the mutants known to disrupt sister-chromatid cohesion (reviewed in Miyazaki and Orr-Weaver, 1994), only two have been shown to encode proteins that localize to chromosomes in metaphase and are thus candidates for proteins that physically constrain sister-chromatids from separating. One is the *S. cerevisiae* mitotic protein Mcd1p/Scc1p, and the other is the Drosophila meiotic protein MEI-S332. In mutants for *scc1*, sister chromatids separate precociously as observed by fluorescence in situ hybridization (FISH) and by tagging chromosomes with GFP (Guacci et al., 1997; Michaelis et al., 1997). In wild-type yeast, sister-chromatid cohesion in mitosis is believed to be established immediately after replication as FISH studies have found that chromatids are not in observably different locations until anaphase. Sister-chromatid cohesion appears to be established appropriately in *scc1* mutants, but it cannot be maintained (Michaelis et al., 1997). Perhaps catenation is sufficient to establish sister-chromatid cohesion immediately after S-phase, and proteins are required to maintain cohesion. Scc1p binds to chromosomes from S-phase until the initiation of anaphase, and it appears that the protein dissociates from sister chromatids at about the time they separate at anaphase (Michaelis et al., 1997). Scc1p is completely degraded by G1 of the next cell cycle (Guacci et al., 1997). *scc1* mutants also cannot fully condense their chromosomes, suggesting that the mechanism of Scc1p action might be indirect (Guacci et al., 1997).

MEI-S332 protein localization and *mei-S332* mutant defects are similar to those of Scc1, but MEI-S332 appears to be required for sister-chromatid cohesion in meiosis and not mitosis. It has already been noted that *mei-S332* mutants cannot maintain centromeric cohesion after anaphase I in spermatocytes (Kerrebrock et al., 1992). In spermatocytes, the MEI-S332 protein is present at the centromeric regions of each pair of sister-chromatids during meiosis until the chromatids separate at anaphase II (Kerrebrock et al., 1995). This study was restricted to spermatocytes because the meiotic cytology was much more accessible than in oocytes. Accordingly, it was not known from this study where MEI-S332 localized in oocytes, with their longer and more highly regulated meiotic cell

cycle. Additionally, from spermatocytes it was not possible to perform Western blots to learn when the protein was degraded.

The question of when sister-chromatid cohesion proteins are degraded is of particular interest because it appears to be under control of cell-cycle regulators. This question has been best studied in mitosis, where it is known that cyclin B is degraded at the end of mitosis by ubiquitin-mediated destruction (Glotzer et al., 1991). More recently, it was discovered that cyclin B is ubiquitinated by a complex called the Anaphase Promoting Complex (APC; King et al., 1995) or cyclosome (Sudakin et al., 1995), a 20S complex containing ubiquitin-conjugating enzymes and regulatory factors. As its name suggests, the APC is also believed to destroy a protein essential for maintaining sister-chromatid cohesion. In experiments in Xenopus mitotic extracts, the addition of APC inhibitors acted to prevent sister-chromatid separation, even though sister chromatids could separate in the presence of non-degradable cyclin B (Holloway et al., 1993). In *S. cerevisiae*, mutations in an APC component also prevented anaphase (Irniger et al., 1995). These experiments predicted the existence of a cohesion-specific substrate of the APC.

These predictions were fulfilled by the discovery that the Pds1p protein of *S. cerevisiae* was degraded by the APC. *pds1* mutants have high rates of chromosome loss at low temperatures and cannot elongate anaphase I spindles at high temperatures (Yamamoto et al., 1996). Additionally they cannot maintain sister-chromatid association as wild-type cells do under a range of conditions: nocodazole treatment, DNA-damage checkpoint arrests triggered by irradiation or mutations, or the mutational inactivation of APC components (Yamamoto et al., 1996). For these reasons, Pds1 was formally considered an inhibitor of anaphase. It appears that Pds1p is not the sole anaphase inhibitor, however, because only about 50% of the *pds1* mutant cells lose sister-chromatid cohesion in these assays (Cohen-Fix et al., 1996). Biochemical experiments demonstrated that Pds1p is ubiquitinated by the APC and is degraded just before the initiation of anaphase in an APC-dependent manner (Cohen-Fix et al., 1996). Thus Pds1p is an important link between the cell-cycle and sister-chromatid separation. Unfortunately, it is not clear whether this is a direct link because although it is known that Pds1p is a nuclear protein, it is not known whether it localizes to chromosomes. One possibility is that Pds1p acts on Scc1p, which physically holds chromosomes together until anaphase. Whether similar mechanisms work in meiosis is unknown.

A functional homolog of Pds1p appears to be the S. pombe gene *CUT2*, although these genes do not share any significant regions of sequence homology.

Cut2p is also degraded in an APC-dependent manner at the start of anaphase and appears to be required for sister-chromatid cohesion (Funabiki et al., 1996). Cut2p localizes to the mitotic spindle, suggesting that it may act by a different mechanism than Pds1 (Funabiki et al., 1996). Also, Cut2p is essential in *S. pombe*, as is expected from a gene required to maintain sister-chromatid association, whereas a null allele of Pds1p does not cause lethality except at high temperatures.

It is clear that proteins play an important role in holding sister chromatids together until anaphase. The mechanism by which they act, however, is unclear. They could bind to chromatin structural proteins, or possibly to DNA itself; they could simply dimerize, with one molecule holding each chromatid, or they could form an elaborate scaffold. It is also conceivable that some cohesion proteins could act by inhibiting access of topo II to DNA, maintaining catenation until the protein is removed.

CONCLUSION

The meiotic cell cycle of animal oocytes must coordinate a number of events. The cell cycle resumes, arrests, and restarts again; it inhibits DNA replication while promoting two rounds of chromosome segregation; and it governs the behavior of the chromosomes to remain attached and to separate at the appropriate times. This coordination requires some meiosis-specific functions, such as mos, *twine*, and MEI-S332, and these have been discovered only through studies of meiosis and oocyte cell cycles. The meiotic cell cycle also utilizes many players from the mitotic cell cycle, such as cdc2, MAPK, and topo II, and accordingly many insights into meiotic processes have come from studies on mitotically dividing cells.

This thesis examines three major aspects of meiosis in oocytes: the regulation of the cell cycle, the mechanisms of sister-chromatid cohesion and release, and the regulation of sister-chromatid cohesion by the cell cycle. In chapter 2, I identify and analyze two mutants, *grauzone* and *cortex*, that are required for the progression of the meiotic cell cycle in oocytes but not spermatocytes. Chapter 3 presents a method for examining the cytology of meiosis in oocytes after metaphase I; this tool is used to investigate the requirements for new protein synthesis during this period. In chapter 4, the localization of the MEI-S332 protein is described in oocytes, and its degradation is examined with respect to the meiotic cell cycle. Finally, in chapter 5, a candidate regulator of MEI-S332 is

presented which may tie the release of sister-chromatid cohesion to the meiotic cell cycle.

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Chapter Two

The Drosophila Genes *grauzone* and *cortex* Are Necessary for Proper Female Meiosis

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ABSTRACT

In Drosophila, normal female meiosis arrests at metaphase I. After meiotic arrest is released by egg activation, the two meiotic divisions are rapidly completed, even in unfertilized eggs. Since little is known about the regulation of the meiotic cell cycle after the meiotic arrest, we screened for mutants that arrest in meiosis. Here we describe the phenotype of eggs laid by sterile mothers mutant for either *grauzone* or *cortex*. These eggs arrest in metaphase of meiosis II, and although they can enter into an aberrant anaphase II, they never exit meiosis. Prolonged sister-chromatid cohesion is not the cause of this arrest, since a premature release of sister cohesion does not rescue the meiotic arrest of *cortex* eggs. Aberrant chromosome segregation at meiosis I was the earliest observable defect, suggesting that *grauzone* and *cortex* are first required immediately after egg activation. The cortical microtubules are also defective, remaining in a pre-activated state in activated mutant eggs. The mutations had no observable effect on either male meiosis or mitosis. We believe these genes will provide insight into the developmental regulation of meiosis in a genetically tractable organism.

INTRODUCTION

Meiosis can be viewed as a variation on mitosis, with an extra round of chromosome segregation allowing the formation of haploid gametes. There are two key differences between the events of meiosis and mitosis. First, meiosis I separates homologs from each other while sister chromatids remain attached, whereas meiosis II and mitosis both separate sister chromatids. Second, during meiosis it is essential to inhibit DNA replication between the rounds of chromosome division. Both meiosis and mitosis share a key regulator, MPF, composed of cyclin B and $p34^{cdc2}$, that was identified independently as a regulator of mitosis in fission yeast and a regulator of meiosis in Xenopus (for reviews, see Coleman and Dunphy, 1994; Murray and Kirschner, 1989).

The timing of the meiotic cell cycle must be coordinated with two key developmental processes: the development of the gamete and the nuclear fusion that occurs at fertilization. This coordination is accomplished in female meiosis by arresting at one or more points in the cell cycle so that events can be synchronized when the arrest is released. In females of most organisms, the meiotic cell cycle arrests for the first time in G2 or prophase I, as the oocyte differentiates and develops, and is released near the end of oogenesis (for reviews see Murray and Hunt, 1993; Sagata, 1996). In many species, meiosis is arrested again, often at metaphase I or II, to wait for fertilization. The molecular regulators of this second arrest are best understood in vertebrate oocytes that arrest at metaphase II. In Xenopus and mouse, MPF controls the timing of the meiotic divisions, increasing at the first meiotic division, falling between the divisions, and increasing again for metaphase II (Gerhart et al., 1984; Kubiak et al., 1992). The metaphase II arrest is dependent upon the stabilization of MPF by an activity known as cytostatic factor (CSF; Masui and Markert, 1971; Murray and Kirschner, 1989), one component of which is the serine-threonine kinase Mos (Sagata et al., 1989). At fertilization, a calcium/calmodulin-dependent event permits degradation of the cyclin component of MPF, releasing the metaphase II arrest (Berridge, 1993; Lorca et al., 1991; Whitaker and Patel, 1990).

An organism with advanced genetics and detailed cytology, such as Drosophila, affords an excellent opportunity for understanding the timing and progression of meiosis in oocytes (reviewed by Orr-Weaver, 1995). After an initial arrest in prophase I to allow for oocyte development, the mature Drosophila oocyte arrests in metaphase I (King, 1970). Mutants defective in

recombination do not arrest at metaphase I, however, implying that recombination is required for the arrest (McKim et al., 1993). The presence of a single crossover between just one pair of homologs that are being pulled to opposite poles is enough to ensure that the cell cycle arrests in metaphase I (McKim et al., 1993). Thus the mechanical tension itself is required to maintain the arrest. TWINE, a homolog of the MPF-activating phosphatase cdc25, is also required since *twine* mutants do not arrest at metaphase I (Alphey et al., 1992; Courtot et al., 1992). In the marine mollusc Patella, which like Drosophila is a protostome that arrests at metaphase I, high levels of MPF accompany the metaphase I arrest, suggesting that the same may be true in Drosophila (Néant and Guerrier, 1988; van Loon et al., 1991).

The meiosis I arrest is released by a process called activation, which takes place as the egg passes through the oviduct into the uterus just before it is fertilized and then laid (Mahowald et al., 1983). Although activation prepares the egg for pronuclear fusion, it is independent of fertilization; and activation, not fertilization, stimulates the completion of meiosis (Doane, 1960; Mahowald et al., 1983). Activation stimulates many events in the oocyte, but the molecular basis of activation is not understood. It has been observed that activated oocytes are slightly swollen, suggesting that hydration may be an important part of the process. Consistent with the hydration model, hypotonic buffers have been found to activate oocytes in vitro (Mahowald et al., 1983). Once activated, eggs complete the two meiotic divisions without delay, and if fertilized, they go on to begin syncitial mitotic divisions. The timing of these events is rapid: it has been estimated that the two meiotic divisions and the first mitotic division all take place within 17 minutes after activation (Foe et al., 1993).

Very little is known about the regulation of the meiotic divisions after the metaphase I arrest in Drosophila oocytes, and it is unclear how much may be assumed by homology to systems with a metaphase II arrest. In order to begin to understand the regulation of the meiotic divisions in Drosophila, we wanted to identify genes required for the proper functioning of those divisions. Our approach was to screen for mutants that cannot complete meiosis. We found two such mutants, *grauzone* and *cortex*, that appear to be involved in the developmental signaling of the completion of meiosis in females.

MATERIALS AND METHODS

Fly Stocks

The five *grauzone (grau)* alleles, *QE70, RG1, QQ36, QF31*, and *RM61*, and two *cortex (cort)* alleles, *QW55* and *RH65*, were generated by Schupbach and Weischaus (1989) in an EMS screen for female sterile loci on the second chromosome and were obtained from T. Schupbach. Since the original chromosomes with $cort^{RH65}$ and $grau^{RM61}$ each carried one or more lethal loci, recombinants were made with a *pr cn bw* chromosome to examine homozygotes. Unfertilized eggs were laid by females mated to *XO* sterile males, who were derived by crossing wild-type males to a compound *X* stock. *Df(2R)Pu-D17*, which uncovers the *grauzone* locus, was obtained from the Bloomington stock center (O'Donnell et al., 1989). Wild-type strains are Oregon R, Canton S, or *yw*. The *cortex mei-S332* doubly mutant chromosome was constructed from the chromosomes *cn* $cort^{QW55}$ *bw* and *cn* $mei\text{-}S332^{7}$ *px sp*. Since *cortex* and *mei-S332* are unlinked, putative recombinant lines carrying *px* as a linked marker for *mei-S332* were tested for the presence of *cortex* and *mei-S332* by complementation with the original chromosomes. Homozygotes of recombinant line 3-19, used for this study, were found to be female sterile but male fertile, giving the *mei-S332* missegregation phenotype in male meiosis. Markers are described in Lindsley and Zimm (1992).

Egg and Embryo Staining

Fertilized and unfertilized mutant eggs, and fertilized or unfertilized wild-type embryos or eggs were collected over the course of 30 minutes, 2 hours, or 3 hours and dechorionated in 50% Chlorox bleach. For spindle staining, eggs were devitellinized, fixed in methanol, and rehydrated by standard methods (Theurkauf, 1994). They were then incubated with a mouse monoclonal ascities fluid antibody against β-tubulin (Amersham), diluted 1:2000 in PBST (130 mM NaCl, 70 mM Na_2HPO_4, and 35 mM NaH_2PO_4 [PBS] with 0.3% Triton X-100). The primary antibody was detected by DTAF-labeled goat anti-mouse (Jackson) diluted 1:250 in PBST. DNA was detected by one of three methods: incubating 20 minutes with 1 mg/ml boiled RNase A and then staining with propidium iodide (Sigma) at 1 µg/ml in PBS for 20 minutes; staining with 5 µg/ml 7-aminoactinomycin D (7-AAD, Molecular Probes) in PBST for 30 minutes; or staining with 1

μg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma) in PBS for 10 minutes. DAPI could not be used with the confocal microscope, and 7-AAD could only be marginally detected, whereas propidium iodide was easily imaged on the confocal microscope. Samples were dehydrated in methanol and mounted on slides in clearing solution (2:1 benzyl benzoate: benzyl alcohol) (Theurkauf and Hawley, 1992) containing 50 mg/ml propyl gallate as an anti-bleaching agent, except for DAPI-stained samples, which were mounted in 70% glycerol/30% PBS.

For visualization of cortical microtubules, laid mutant and unfertilized wild-type eggs were fixed at the interface of 37% formaldehyde and heptane for 10 minutes; and either devitellinized in methanol, or manually devitellinized and permeabilized in 1% Triton X-100 for 2 hours as described by (Theurkauf, 1994). Devitellinized eggs were rehydrated and stained as above for spindles.

Determination of an Aberrant Meiosis I

Meiosis I defects were difficult to diagnose with certainty in meiosis II eggs because only rarely could all 16 chromatids be distinguished. Eggs were considered to have an aberrant meiosis I only if they met one of the following three conditions: 1) they arrested in meiosis I; 2) too many chromosomes were visible on one meiosis II spindle; or 3) a third meiotic spindle was visible, and it contained chromosomes missing from both of the other two spindles. It was also rare that we could determine with certainty that chromatids had segregated normally in meiosis I. The difficulty of assigning interpretation to these meiosis II figures means that although the minimum meiosis I defect is 30%, the actual rate of aberrant meiosis I events could be substantially higher.

Ovary and Oocyte Cytology

To examine ovaries, females of each of the five *grau* alleles in trans to *Df(2R)Pu-D17* and of the two *cortex* alleles in trans to each other were fattened 3-10 days on yeast, and dissected in Drosophila EBR (129 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM HEPES pH 6.9), fixed in 8% formaldehyde in EBR for 5 minutes, and stained with DAPI as described for eggs and embryos above. The morphology of ovaries was compared between mutant females and their heterozygous sisters.

To examine oocytes, we collected, fixed, and manually dechorionated oocytes *en masse* with the blender method described by Theurkauf and Hawley (1992). After extraction in 1% Triton X-100 for two hours, the DNA was labeled

either with propidium iodide as for embryos above, or with a monoclonal anti-histone antibody (Chemicon) diluted 1:500 and detected by a Texas Red-conjugated anti-mouse antibody (Jackson) diluted 1:250. Tubulin was labeled with two rat monoclonal anti-tubulin antibodies, YL1/2 and YOL1/34 (both from Sera-Lab) each diluted 1:5 and detected by a DTAF-conjugated anti-rat antibody (Jackson) diluted 1:100. All dilutions were in PBST, and antibody dilutions also included 0.1% BSA. Samples were mounted in clearing solution, as above. The stage of oogenesis was determined by the nuclear and spindle morphology.

Microscopy

Samples were examined and scored with a Zeiss Axioskop microscope with 5X and 40X dry Plan Neofluar objectives. Images were captured with a Bio-Rad MRC 600 confocal laser scanning head equipped with a krypton/argon laser, mounted on a Zeiss Axioskop microscope. Alternatively, confocal images were captured on a Zeiss LSM410 mounted on a Zeiss Axiovert microscope. Images were taken with 20X, 40X oil, and 63X oil Plan Neofluar objectives. In some cases, optical sections were taken and projected into a single plane. In order to avoid bleed-through from one channel into another, we found it necessary to excite singly at 488 and 568 nm. Since 7-AAD is not optimally excited by the confocal laser, to image 7-AAD-stained nuclei we used a Photometrics Image Point Cooled CCD video camera installed on a Nikon Optiphot-2 microscope, using a 40X oil Nikon objective and a variable zoom lens. Images were processed, colorized, and merged on a Macintosh Power PC with the program Adobe Photoshop.

Male Meiotic Segregation Tests

Tests for meiotic sex-chromosome segregation in males were performed essentially as described in (Kerrebrock et al., 1992). Briefly, stocks were constructed with each allele of *grau* and *cort* homozygous in a background with *y* on the X chromosome and y^+ marking the Y chromosome; stocks were also constructed with each *grau* allele in trans to *Df(2R)Pu-D17*, and with the *cort* alleles in trans to each other, in the same y/y^+Y background. To determine levels of meiotic missegregation in these males, they were crossed to virgin *C(1)RM*, y^2 $su(w^a)$ w^a. The presence of the marked attached-X chromosomes in the females permitted recovery of progeny from normal male gametes as well as gametes lacking the sex-chromosomes or carrying two copies of the sex chromosomes. Thus the frequency of sex-chromosome missegregation could be calculated.

Parents were removed before the progeny eclosed, and progeny were scored before eclosion of the F2 generation.

RESULTS

Mutations in the *grauzone* and *cortex* genes cause arrest in meiosis II

We were interested in identifying genes that regulated or participated in the meiotic cell cycle. Reasoning that mutations in some meiotic genes might cause meiosis to fail entirely and lead to sterility, we screened through several collections of Drosophila female sterile mutants to find meiotic mutants (Berg and Spradling, 1991; Cooley et al., 1988; Lehmann and Nusslein-Volhard, 1986; Mohler and Carroll, 1984; Mohler, 1977; Schupbach and Wieschaus, 1989; Zalokar et al., 1975). In wild-type eggs, the two meiotic divisions take place within the dorsal anterior ooplasm after oogenesis, and all four meiotic products are visible inside the egg by the time it is laid. In order to examine the meiotic products in eggs from mutant females, eggs from 0-2 hour collections were fixed and stained with the DNA stain DAPI. Wild-type eggs in a collection of this length nearly always show the multiple nuclei typical of the syncitial mitotic divisions and have visible polar bodies as evidence of the completion of meiosis. In contrast, mothers mutant for either of two genes, *grauzone* or *cortex*, laid eggs that typically had 2-4 sets of condensed chromosomes in the dorsal anterior quadrant of the egg, suggesting an arrest in meiosis. Out of approximately 100 female sterile lines screened, many of which were preselected for early arrest of embryonic development, only two lines, *grau* and *cort*, arrested in meiosis. Five recessive alleles of *grau* and two recessive alleles of *cort* were generated in the Schupbach screen, an EMS screen for female sterile mutations (Schupbach and Wieschaus, 1989).

To analyze the meiotic arrest more fully, we used immunofluorescence to examine the spindles and chromosomes in eggs from *grauzone* and *cortex* mothers, hereafter called *grau* and *cort* eggs. The presence of two major spindles in 80-90% of mutant eggs indicated an arrest in meiosis II (Fig. 2-1A-C), whereas one major spindle in a small percentage of eggs indicated a meiosis I arrest (Table 2-1). About a third of the eggs had chromosomes that had moved off the major spindles and organized their own tiny spindles, and in a small percentage of these, the chromatin was so disorganized that it was difficult to classify the arrest as meiosis I or II. Mutant eggs were fertilized, as evidenced by staining with antibody AX-D5 which recognized the sperm tail within the egg (Karr, 1991) data not shown). The inappropriate meiotic arrest appeared identical in both fertilized and unfertilized mutant eggs (data not shown), which was expected since unfer-

Figure 2-1. Cytology of eggs from *grauzone, cortex,* and wild-type mothers.

Red represents DNA and green is anti-tubulin immunofluorescence. A,B,C, and E are stained with propidium iodide, and D with an anti-histone antibody. Scale bar in A is 25 μm; scale bars in B,C,D, E are 10 μm.

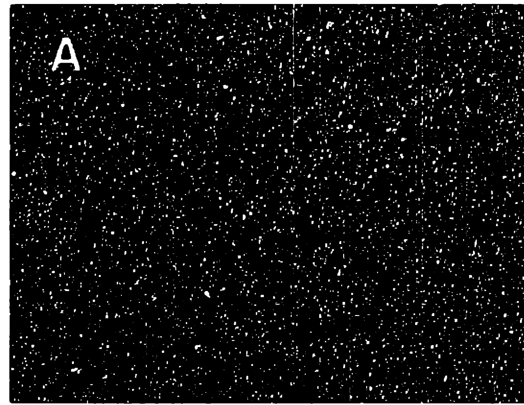
A) The anterior half of an egg from a *cortex*^{QW55} mother showing two spindles arrested in metaphase II.

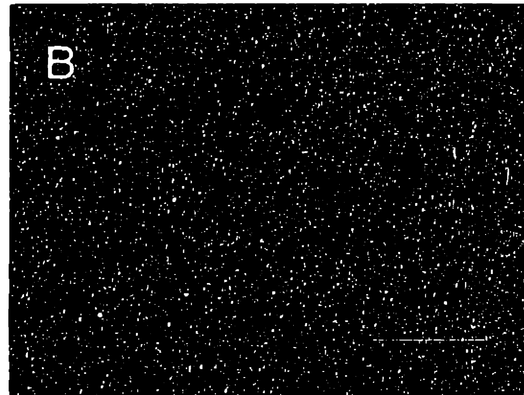
B) Spindles from an egg laid by a *grauzone*^{QQ36} mother, arrested in metaphase II.

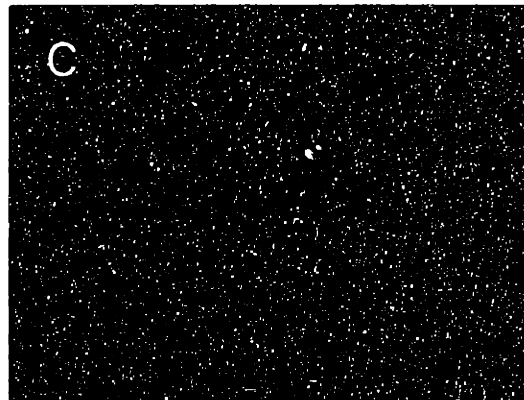
C) Spindles from an egg laid by a *grauzone*^{QQ36} mother, arrested in anaphase II.

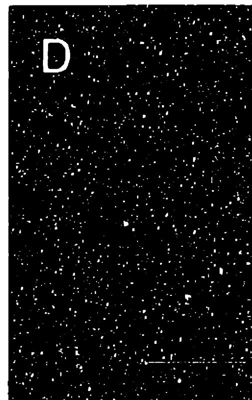
D) A normal metaphase I-arrested spindle from a wild-type oocyte.

E) Normal polar bodies from an unfertilized wild-type egg, with three meiotic products condensed to form a bouquet structure.









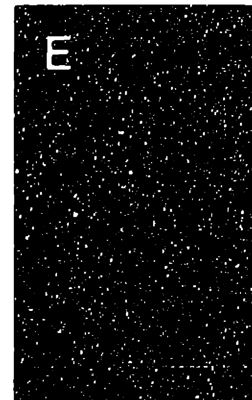


Table 2-1.

Phenotypes of eggs laid by *grau* and *cort* mothers

maternal genotype	meiosis I arrest	meiosis II arrest	meiosis I or meiosis II arrest[a]	completed meiosis	total eggs examined[c]
grau^{QE70}	14%	84%	2%	0	116
grau^{QE70}/ *Df(2R)Pu-D17*	2%	82%	10%	0	100
grau^{RG1}	3%	89%	3%	0	36
grau^{RG1}/ *Df(2R)Pu-D17*	2%	81%	6%	0	48
cort^{RH65}	3%	89%	6%	0	102
cort^{QW55}	0	82%	9%	7%[b]	45
wild-type (CantonS)	0	0	0	100%	150

[a]Eggs with one major spindle and one or more minor spindles could represent either an aberrant meiosis I or an aberrant meiosis II, and so could not be categorized.

[b]In *cort*^{QW55} eggs, in each case where meiosis was completed, the egg arrested with aberrant polar bodies and did not develop.

[c]Less than 2% of the total eggs were excluded from the tabulations because although they failed to complete meiosis, they had too many meiotic spindles or other cell cycle defects, and so could not be categorized.

tilized wild-type eggs complete meiosis (Doane, 1960). In general, the meiotic arrest phenotypes in eggs from *grau* mothers and *cort* mothers were cytologically indistinguishable.

The alleles of *grauzone* all appeared to be approximately equivalent in strength, since eggs from homozygous mothers of the five alleles had similar phenotypes. We examined eggs from mothers hemizygous for each *grau* allele, that is, that had each allele of *grau* in trans to a deficiency that uncovers the *grau* locus, *Df(2R)Pu-D17*. The range of phenotypes in these eggs was similar to eggs from homozygous mothers (Table 2-1 and data not shown). The equivalence of the hemizygous and homozygous phenotypes is consistent with, but not proof of, these mutations being null alleles. The two alleles of *cortex* had slightly different phenotypes, since $cort^{QW55}$ eggs did occasionally complete meiosis, whereas $cort^{RH65}$ never completed meiosis (Table 2-1). This suggests that *RH65* is a stronger allele than *QW55*. Despite the outcrossing of the RH65 chromosome (see Materials and Methods), it is formally possible these differences could be caused by a modifier on chromosome 2. Unfortunately, we were unable to locate a deficiency that uncovers *cortex*.

The spindles seen in mutant eggs were clearly arrested in the meiotic divisions rather than in the normal meiotic arrest or in mitosis. In addition to the prophase I arrest of immature oocytes, mature Drosophila eggs have two cytologically-defined normal arrest states: one arrest is at metaphase of meiosis I (Fig. 2-1D), and it is released at egg activation as the egg is laid; and the other is a post-meiotic arrest where the three or, if the egg is unfertilized, four unused meiotic products condense. These condensed chromosomes are pulled together into 1-3 bouquet structures on the dorsal surface of the egg where they persist for the first several syncitial divisions (Fig. 2-1E). The terminal arrest state of *grauzone* and *cortex* mutant eggs was not at either of these two normal arrest states, but rather during the meiotic divisions. The mutants arrested after metaphase I, as judged by the presence of two spindles or by the separation of chromosomes in cases where there was only one spindle (although the metaphase I arrest was normal, see below). The mutant eggs were arrested at a later stage of meiosis and not in mitosis as demonstrated by two lines of evidence. First, Drosophila meiotic and mitotic spindles have different morphologies in that mitotic spindles have asters at their ends and meiotic spindles are tapered and narrow at the poles (Theurkauf and Hawley, 1992). The spindles seen in mutant eggs from short collections are tapered like meiotic spindles, and although older eggs from longer collections

have thickened and blunted spindles, they still lack asters at the poles (data not shown). Secondly, *grau* and *cort* eggs do not have meiotic products or bouquet structures, demonstrating that they have not completed their meiotic divisions.

The meiosis II-arrested eggs appeared to be arrested at both metaphase II and anaphase II. To understand this dual-arrest point, we compared eggs from short collections to those from long collections. About 60% of the eggs examined in a 1/2-hour collection were arrested in metaphase II (Fig. 2-1B), whereas in 2-hour collections the eggs were more often arrested in anaphase II, with only 15% still in metaphase II. This demonstrated that although the initial arrest was in metaphase of meiosis II, over time the chromosomes could separate into anaphase II. This anaphase II was sometimes aberrant, with unequal numbers of chromosomes segregating to each pole, with high numbers of lagging chromosomes, and with chromosomes sometimes highly separated from one another and never reaching the poles (Fig. 2-2). In the older eggs the spindles appeared larger and thicker than in younger eggs, and many show evidence of having undergone anaphase B movements. In the longer collections we never saw chromosomes decondensing into telophase II. Thus eggs from mutant *grauzone* and *cortex* mothers arrest at metaphase II. Although this arrest eventually breaks down, the eggs never complete and exit meiosis.

The earliest defect in *grau* and *cort* mutants is in anaphase I

Although *grauzone* and *cortex* mutant eggs arrested in meiosis II, it was possible that the initial defect took place at some earlier time: in oogenesis, at the metaphase I arrest, or in the first meiotic division. In order to determine whether oogenesis proceeded normally in *grau* and *cort* females, we stained the DNA of dissected ovaries from females hemizygous for all *grau* alleles and from $cort^{RH65}/cort^{QW55}$ females. Most ovaries had completely normal morphology. We observed occasional pycnotic nurse cell nuclei in a minority of ovaries from two *grau* mutants, alleles *RG1* and *QF31*, but these few defective egg chambers were in separate normal ovarioles. Because the low penetrance of the nurse cell defect was incommensurate with the high penetrance of the meiosis II arrest, we believe that these are either background effects or weak pleiotropies of these *grau* mutations.

To determine whether there was a defect in the metaphase I arrest in *grauzone* and *cortex* mutant oocytes, we examined the spindles and chromatin of stage 14 oocytes, in the last stage of oogenesis when the metaphase I arrest occurs,

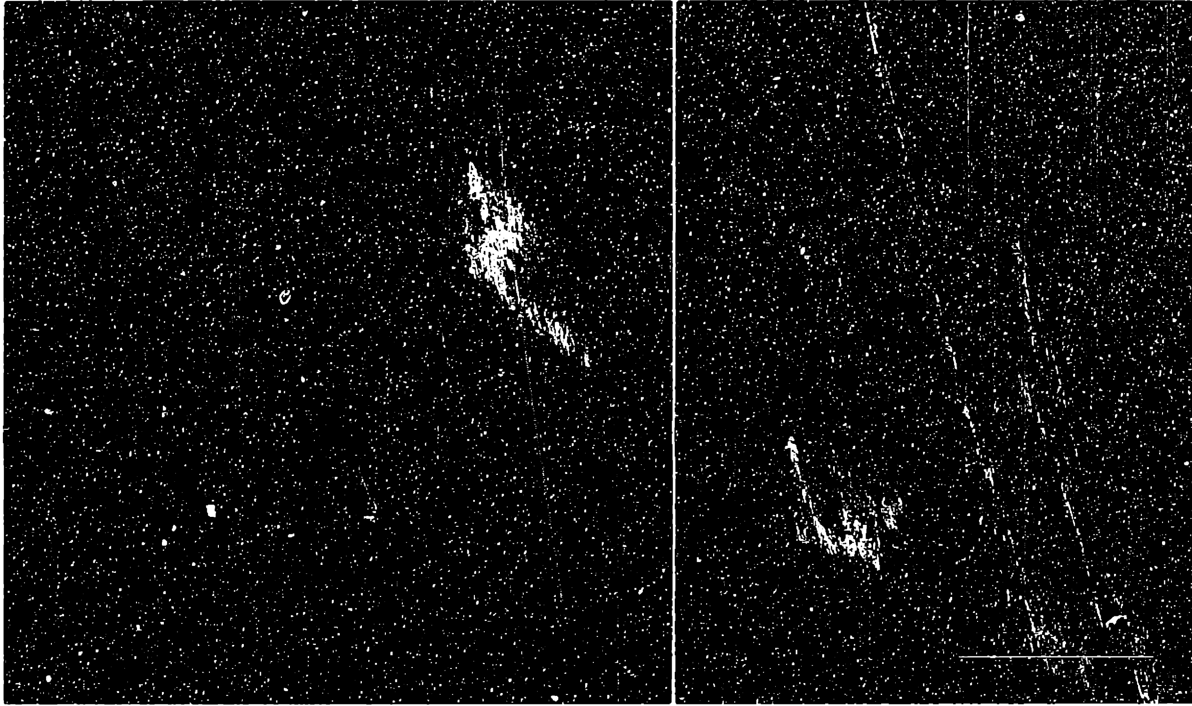


Figure 2-2. Two spindles from the same egg, laid by a *grauzone*^{RG1} mother.

Red is propidium iodide staining of DNA, and green is anti-tubulin immunofluorescence showing the meiotic spindle. The chromosomes are spread throughout the spindles in aberrant anaphase II. The spindle on the left has 11 of 16 total chromatids, and the spindle on the right has 5, indicating that meiosis I chromosome segregation was unequal. The spindles were in different focal planes. Scale bar is approximately 5 μm.

and compared mutant oocytes to heterozygous sibling oocytes and wild-type stage 14 oocytes. Normally the stage 14 oocyte nucleus is arrested with the chromosomes condensed on a meiosis I spindle (Fig. 2-1D). In older metaphase I-arrested wild-type oocytes, the chromatin becomes elongated and the tiny fourth chromosomes move toward the poles. *grau* and *cort* metaphase I arrested oocytes had normal cytology (Fig. 2-3). Thus nearly all *grau* and *cort* eggs appeared to develop normally through the metaphase I arrest.

The metaphase I arrest is released at egg activation. If there were a defect in the metaphase/anaphase transition triggered by egg activation, that might be evidenced by subsequent defects in the first meiotic division. Indeed, in addition to the ~10% mutant eggs that may be arrested in meiosis I (Table 2-1), immunofluorescence of spindles and chromosomes showed that many eggs have unequal numbers of chromosomes in the two meiosis II spindles, a consequence of unequal segregation in meiosis I (Fig. 2-2). By counting chromosomes, we found that on average 27% of the first meiotic divisions were visibly aberrant (in 329 *grau* and *cort* eggs), although the actual rate of meiosis I missegregation may have been substantially higher (see Materials and Methods). Thus, although the metaphase I-arrested oocyte appears normal, observable defects begin as soon as the arrest is released.

cortex mutants are not rescued by premature separation of the sister chromatids

The meiotic arrest in metaphase II raised the possibility that the primary defect in the mutants was a failure to release sister-chromatid cohesion at the metaphase II/anaphase II transition. The sister chromatids are physically attached to each other prior to anaphase II, and the release of this cohesion is necessary for anaphase movement to occur (reviewed by Bickel and Orr-Weaver, 1996). Inappropriate maintainance of sister-chromatid cohesion along the chromosome arms might also stabilize the attachment between homologs in meiosis I and prevent their timely release at anaphase I (Bickel and Orr-Weaver, 1996); so a defect that prolonged sister chromatid-cohesion could also cause the missegregation of homologs that we observed in meiosis I.

A direct way to test the possibility that *grau* and *cort* mutants fail to release the sister cohesion is by separating the sister chromatids early, before metaphase II, and observing whether this can rescue the meiotic arrest. The Drosophila gene *mei-S332* is required to hold sister chromatids together until anaphase II, and

Figure 2-3. Metaphase I in *grau*^{QE70} (A) and *cort*^{RH65} (B) appears normal. Mutant stage 14 oocytes were fixed in formaldehyde, the DNA was stained with propidium iodide (red), and the spindles were stained with anti-tubulin (green). In (B) the spindle is more advanced, with the fourth chromosomes (yellow) visible between the chromo-some mass and the poles (see Theurkauf and Hawley, 1992). Scale bar is 5 μm.

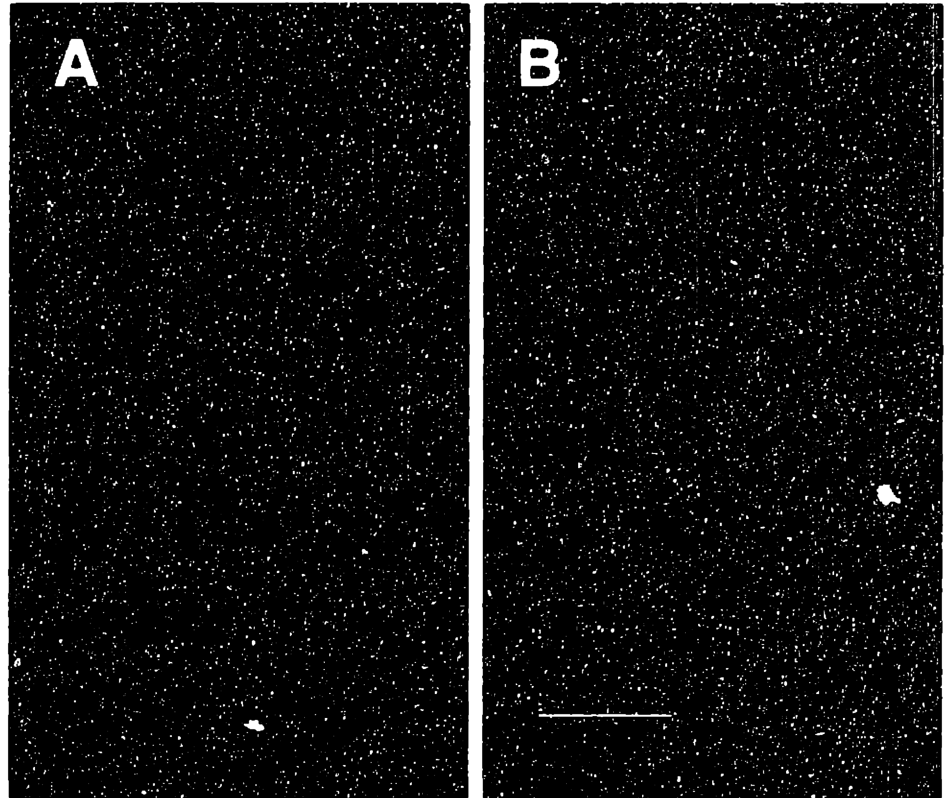
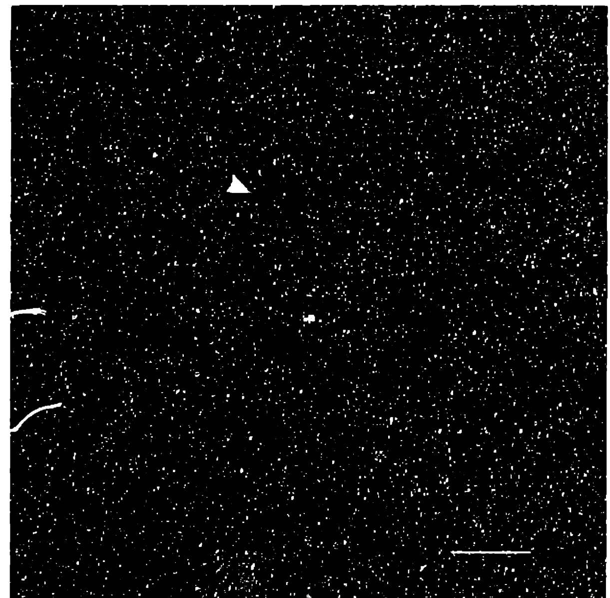


Figure 2-4. *cort mei-S332* eggs arrest in meiosis.

Double mutant eggs were fixed in methanol and the DNA was stained with 7-AAD (red) and the microtubules were stained with anti-tubulin (green). The sister chromatids have separated, since more than 8 chromosomes can be identified. Many chromatids have organized tiny spindles (example shown with arrow). Scale bar is 10 μm.



mutations in *mei-S332* cause premature separation of the sister chromatids at the end of anaphase I (Davis, 1977; Goldstein, 1980; Kerrebrock et al., 1992; Kerrebrock et al., 1995). *mei-S332* homozygous flies are fertile, and their eggs complete meiosis and make normal polar body structures. We constructed the double mutant *cortex mei-S332* and tested whether doubly mutant homozygotes laid eggs that arrested in meiosis or instead proceeded into zygotic development. We did not construct the *grauzone mei-S332* double mutant because the genetic distance between the loci is very small.

cortex mei-S332 homozygous females were sterile. When we examined the chromosomes and spindles in their laid eggs, we found that chromosomes were scattered throughout the area in which meiosis usually occurs, and that many chromosomes had organized tiny spindles (Fig. 2-4). It has been previously observed that when a chromosome strays from a meiotic spindle, it organizes its own tiny spindle (Theurkauf and Hawley, 1992). No zygotic development was observed in the doubly mutant eggs, and there was no evidence of any mitotic spindles or of polar body structures. This cytology is evidence that the double mutants are still in a meiotic state. We counted more than 8 chromatids in these eggs, indicating that the sister chromatids had separated by the time of the arrest. Thus, the premature separation of sister chromatids did not rescue the meiotic arrest of *cortex* eggs.

Male meiosis is normal in *grauzone* and *cortex* mutants

To address the question of whether *grauzone* and *cortex* might be general meiotic regulators or contribute to meiotic segregation functions that affect meiosis in both sexes, we examined males for meiotic defects. Fertility tests demonstrated that males homozygous mutant for all alleles of *grau* and *cort* are fertile, as are males transheterozygous for *grau* over the deficiency *Df(2R)Pu-D17*. Thus these males did not have an equivalent phenotype to their homozygous mutant sisters, who were isolated in a screen for female sterile flies. Since it was still possible that these males had defects in segregating their chromosomes that could not be detected in the fertility tests, we examined whether they could faithfully segregate the sex chromosomes using standard meiotic nondisjunction tests (see Materials and Methods). We tested $cort^{RH65}/cort^{QW55}$ males and hemizygous *grau* males with each allele of *grau* in trans to the *Df(2R)Pu-D17* deficiency chromosome, and compared them to their heterozygous brothers. This sensitive test detected no missegregation above the levels in heterozygous deficiency

controls (data not shown), and so we conclude that *grauzone* and *cortex* are not required for male meiosis.

grauzone and _cortex_ are not essential for mitosis

If *grauzone* and *cortex* were acting directly on the chromosomes or on the spindle apparatus, they might be expected to be required for mitosis. However, these alleles of *grau* and *cort* were isolated in a screen that required at least some female homozygotes to be viable. Thus it is possible that an allele that demonstrated a role in mitosis would not have been isolated. Nonetheless, not only were all alleles of both genes completely viable as homozygotes, but also all alleles of *grau* were completely viable in trans to the deficiency (data not shown). Consistent with normal viability, we did not observe any visible mitotic defects, including rough eyes, missing or thin bristles, or small body size. *grau* fully complemented all existing lethal loci in the region uncovered by the deficiency *Df(2R)Pu-D17* (data not shown); because no deficiency was available for *cort*, we were unable to do complementation analysis. Although it is still possible that *grau* or *cort* plays a role in mitosis, we find it unlikely that either is an essential component in most cells.

Cortical microtubule fibers are stabilized in _grauzone_ and _cortex_ eggs

grauzone and *cortex* eggs fixed with formaldehyde rather than methanol revealed another defect: the microtubules in the cortex of the eggs were fibrous, compared to wild-type eggs where the tubulin was in a relatively unpolymerized state. W. Theurkauf has found that wild-type stage 14 oocytes have long fibrous microtubules, visible by confocal microscopy at the oocyte cortex (Theurkauf et al., 1992). These fibers depolymerize after egg activation, so that in both mitotically dividing embryos and in unfertilized laid eggs, the cortical microtubules have cleared from the cytoplasm (W. Theurkauf, personal communication). When we stained wild-type stage 14 oocytes and activated, unfertilized laid eggs, we also observed this transition in the cortical microtubules (Fig. 2-5A,B).

In *grauzone* and *cortex* eggs, the microtubules were visible as fibers during stage 14 of oogenesis (Fig. 2-5C,E). They failed to clear from the cytoplasm in response to egg activation, however, and instead persisted during the mutant meiosis II arrest (Fig. 2-5D,F). We found these aberrantly persistent cortical microtubule fibers in the four *grau* alleles we checked and in both alleles of *cort*. Similar fibers were seen in three separate experiments.

Figure 2-5. The cortical microtubules in *grauzone* and *cortex* eggs do not respond properly to activation.

Eggs were fixed with formaldehyde and stained with anti-tubulin. Scale bar is 10 µm.

A) Wild-type stage 14 oocytes with visible microtubule fibers before activation.

B) A wild-type activated, unfertilized egg without microtubule fibers; fibers have cleared from the cytoplasm at activation.

C) A $grauzone^{QE70}$ stage 14 oocyte.

D) A $grauzone^{QE70}$ laid egg in which the microtubule fibers have not cleared after activation.

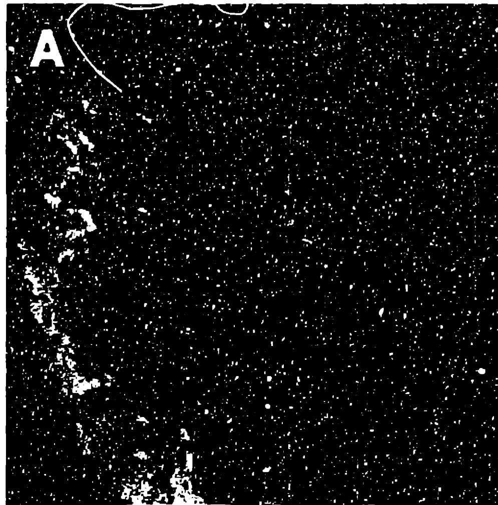
E) A $cortex^{RH65}$ stage 14 oocyte.

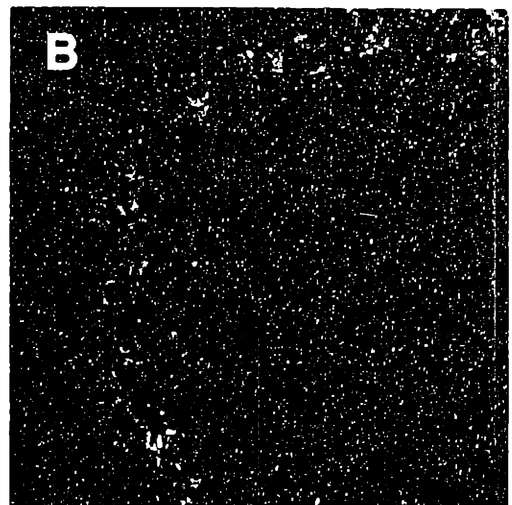
F) A $cortex^{RH65}$ laid egg in which the microtubule fibers have not cleared.

Stage 14 Oocyte

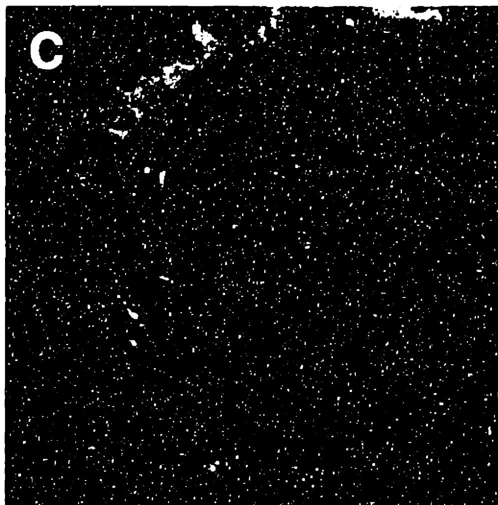
Laid Egg

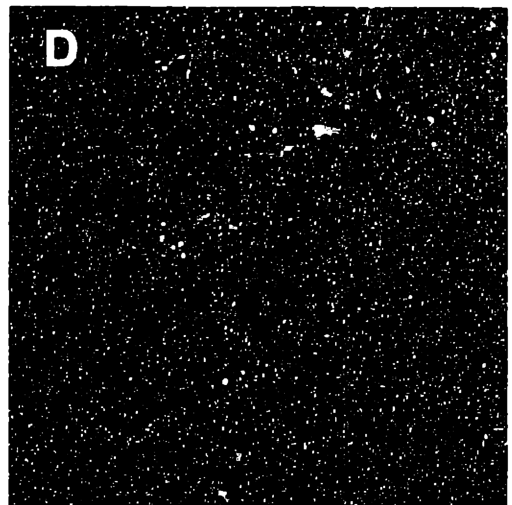
Wild Type



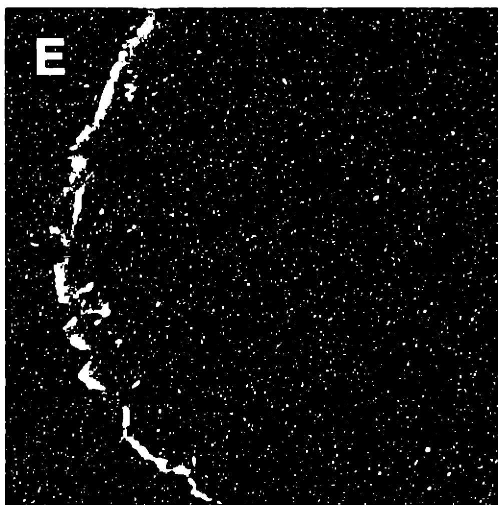


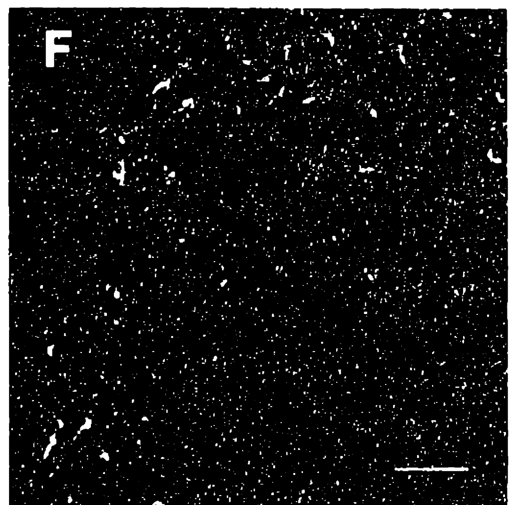
grauzone





cortex





DISCUSSION

grauzone and *cortex* are necessary for the proper continuation and completion of meiosis in Drosophila females. In eggs from mutant mothers, although events up to and including the meiosis I metaphase arrest appear to be normal, the segregation of chromosomes is often unequal in the first meiotic division. Meiosis then arrests aberrantly at metaphase II. Although over time the chromatids on the meiosis II spindles seem to be able to separate from each other, they do not reach the poles and do not enter telophase. In the cytoplasm, short microtubules characteristic of late oogenesis fail to clear from the cortical regions of the laid mutant eggs, and instead persist as short fibers throughout the meiotic arrest.

These genes are intriguing because this meiotic arrest phenotype has not been previously described. Although mutations in the Drosophila *deadhead (dhd)* gene give a maternal effect phenotype that has been reported as a meiotic arrest (Salz et al., 1994), we observed that *dhd* eggs arrested after the completion of meiosis (unpublished results). Therefore the *dhd* gene appears to be required after meiosis. We know of no other Drosophila genes that are required for the progression of female meiosis after the metaphase I arrest. Indeed, to our knowledge no other mutations in other organisms cause a metaphase II arrest, and so we believe that an analysis of these two genes will be useful for understanding the meiotic cell cycle.

Despite the meiotic arrest in metaphase II, *grauzone* and *cortex* are not solely required as part of the machinery that separates sister chromatids at the metaphase II/anaphase II transition. Cohesion between sister chromatids at metaphase II requires the gene *mei-S332* (Davis, 1977; Goldstein, 1981; Kerrebrock et al., 1992; Kerrebrock et al., 1995). In *mei-S332* mutant eggs sister chromatids separate before metaphase II, disturbing the segregation of the chromatids, but then exit meiosis and enter the mitotic divisions. We show here that in *cortex mei-S332* double mutants the sister chromatids have separated but remain in a meiosis-like state. Thus the meiosis II arrest in *cort* eggs is not caused by the prolonged attachment of sister chromatids, since relieving the attachment of sister chromatids does not allow the cell cycle to resume in *cort* eggs. Additionally, it is unlikely that *grau* and *cort* are components of the chromosome segregation machinery in meiosis II, since we would expect such functions to be

conserved between male and female meiosis, and between meiosis and mitosis. Since there is no phenotype in male meiosis or in mitosis, we conclude that *grau* and *cort* must be required for functions specific to oogenesis and female meiosis.

While this work was in progress, we learned that Lieberfarb et al. (1996) had observed another phenotype in *cortex* eggs. They observed that the BICOID protein gradient in the anterior end of embryos was absent in *cort* eggs; we repeated these results and found that the BICOID gradient was also absent in *grauzone* eggs (see Appendix 2). Although they proposed that translation of BICOID was inhibited in *cort* eggs, existing antibodies do not permit quantitation of BICOID protein levels on Western blots. Therefore they, and also we, examined levels of TOLL protein, which like BICOID is translationally regulated by polyadenylation (Sallés et al., 1994). In contrast to their results, we repeatedly find TOLL protein levels to be unaffected in both *grau* and *cort* mutant eggs, as assayed by Western blotting protein extracts of four *grau* mutants and both *cort* mutants (see Appendix 2). Additionally, in collaboration with B. Edgar (Fred Hutchinson Cancer Center), we observed that the level of the cell cycle regulator protein STRING, which is translated de novo after egg activation, is also unaffected in *grau* and *cort* mutant eggs (unpublished results). Thus we do not find convincing evidence of a generalized block in translation. Moreover, the completion of meiosis in Drosophila females does not require protein synthesis after the metaphase I arrest (Page and Orr-Weaver, 1997), demonstrating that meiotic arrest in *cort* and *grau* eggs cannot be ascribed to a block in translation at this time.

In considering possible roles for *grauzone* and *cortex* in female meiosis, how can we reconcile the meiosis I missegregation, the meiosis II arrest, and the persistence of the cortical microtubules? We imagine three possible models: 1) incomplete activation of oocytes; 2) inappropriate cell cycle regulation; and 3) defects in microtubule structure. For the first model, we note that egg activation leads to a number of events in the egg, including swelling of the egg, cross-linking of the vitelline membrane (Mahowald et al., 1983; Spradling, 1993), the completion of meiosis (Doane, 1960), depolymerization of the cortical microtubules (W. Theurkauf, personal communication), and translational activation (Mahowald et al., 1983). It is clear that some events of egg activation do happen normally in the mutant eggs because the vitelline membrane becomes impermeable to bleach (i.e. becomes cross-linked), and the eggs swell (data not shown). However, other events do not occur, such as the depolymerization of the cortical

microtubules. Still other egg activation events happen part-way, e.g., meiosis is resumed but not completed, and translation is blocked for some but not all messages activated at this time. If we conceptualize egg activation as an initial event that starts myriad parallel pathways, then perhaps in the mutant eggs some of those pathways proceed while others are blocked. *grau* and *cort* would be required to connect activation with segregation at meiosis I, the metaphase II/anaphase II transition, exit from meiosis, and depolymerization of the microtubules.

The second model invokes meiotic regulators from other animals. In most organisms that arrest in metaphase I or II of meiosis, the arrest is maintained by stabilizing MPF levels (see Sagata, 1996). In Xenopus oocytes, CSF is responsible for stabilizing MPF; yet at activation (or fertilization) MPF is inactivated before CSF . Although it was initially assumed that CSF inactivation was required to allow the oocyte to progress into anaphase II, it is now believed that CSF inactivation is required so that the egg does not arrest at the *next* metaphase, in the first mitotic division (Watanabe et al., 1991). If similar factors are present in Drosophila, it is possible that *grauzone* and/or *cortex* may be negative regulators of a CSF-like factor. In the mutant eggs, the inappropriate persistence of CSF would cause an arrest at the next metaphase, here at metaphase II. Additionally, we speculate that without negative regulation, CSF might be overexpressed at metaphase I, and could account for the defects in meiosis I. Since MPF is known to affect microtubule structure, its inappropriate stabilization in metaphase II could account for the persistent cortical microtubules (Belmont et al., 1990). The cytology of the metaphase II arrested chromosomes described here is consistent with metaphase-arrested chromosomes caused by the inappropriate stabilization of MPF in Drosophila and other organisms: chromosomes move into aberrant anaphase, remain highly condensed on a stable spindle, and are unable to exit mitosis (Rimmington et al., 1994; Surana et al., 1993). When constitutive MPF activity has been induced in Xenopus egg extracts, the nuclei arrest at a prolonged metaphase, and then enter into an abnormally extended anaphase (Holloway et al., 1993). These phenotypes are similar to the meiosis II arrest that we observe in *grauzone* and *cortex* eggs.

The third model centers around microtubules. If *grauzone* and *cortex* were required directly or indirectly for regulation of microtubule structure, perhaps that would explain the meiotic arrest and the persistent microtubules. Although the meiosis I and meiosis II spindles appear to have normal morphology, they

may not be fully functional. This could explain the missegregation at meiosis I and the arrest at meiosis II . Alternatively, the arrest at meiosis II could be caused by a checkpoint triggered either by the meiosis I aberrant segregation or by a spindle assembly defect. In either model, the cortical microtubules would also be subject to regulation by *grau* and *cort*.

We have postulated that *grauzone* and *cortex* may be responsible for transmitting part of the activation signal, negatively regulating CSF, or regulating microtubule structure. These models are not mutually exclusive because molecular regulators can be involved in many processes. Regardless of the molecular nature of these defects, the existence of these mutants is informative about the meiotic cell cycle in Drosophila and other systems. The mutant phenotype demonstrates for the first time that in Drosophila oocytes the two meiotic divisions must be coordinately regulated, since both divisions are defective in the mutant eggs. Additionally, the sex-specificity of these mutants supports the surprising idea that meiosis II is regulated differently in male and female Drosophila. A molecular analysis of these genes will be useful to understanding the molecular underpinnings of these observations.

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Chapter Three
Activation of the Meiotic Divisions in Drosophila Oocytes

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ABSTRACT

Key meiotic events in many organisms are controlled at the translational level. In this study, we examined the role of translational regulation in the meiotic cell cycle of Drosophila, an organism amenable to genetic analysis. In order to address this question, we developed a system for activating Drosophila oocytes in vitro. With this method, hundreds of mature oocytes can be activated to resume and complete meiosis. The stages of meiosis are normal by cytological criteria, and the timing of the meiotic divisions is similar to that of eggs activated in vivo. We use this system to examine the role of protein synthesis in regulating the progression of meiosis and the maintenance of the metaphase I arrest. We find that synthesis of new proteins after metaphase I is not required for anaphase I, meiosis II, or the decondensation of the meiotic products. Also, continued protein synthesis is not required to maintain the metaphase I arrest. New protein synthesis is required, however, for proper chromatin recondensation after meiosis.

INTRODUCTION

Meiosis is a developmentally regulated cell cycle, in which two rounds of chromosome segregation occur without an intervening S phase. Control of the meiotic cell cycle varies widely in different organisms. Many animals employ developmental arrests of the meiotic cell cycle in order to synchronize the completion of meiosis with the growth of the oocyte and with sperm entry (reviewed in Sagata, 1996; Page and Orr-Weaver, 1997). Since these arrest points occur at different times in the meiotic cell cycle in different species, it is difficult to know how much regulation is conserved. It appears, however, that in many organisms key meiotic events are regulated translationally. In Xenopus, synthesis of new proteins is required for release from the prophase arrest, and for entry into meiosis II (Gerhart et al., 1984; Wasserman and Masui, 1975). In clams and starfish, new proteins are required for entry into meiosis II (Hunt et al., 1992; Picard et al., 1985). Continual synthesis of new proteins is required in the mollusk Patella for maintenance of the meiosis I arrest (Néant and Guerrier, 1988). Since synthesis of cyclin B is required for canonical mitotic divisions, it appears that cell cycle events are often regulated translationally in both meiosis and mitosis.

Most of the current understanding of the meiotic cell cycle has come from studies on biochemically tractable oocytes such as those from Xenopus and clams. These organisms have been ideal for such studies because it is possible to isolate large numbers of oocytes arrested before meiosis, and then to mature them in vitro to enter meiosis. A genetic approach would be an important complement to these studies, and we are thus interested in understanding regulation of the meiotic cell cycle in Drosophila.

Fully grown Drosophila oocytes arrest at metaphase I during meiosis. Held inside the ovary, arrested oocytes are activated as they pass through the oviduct, just before they are fertilized and laid (Mahowald et al., 1983). Activation affects many processes in the oocyte: translation of some messages is initiated, meiosis is resumed, and the microtubules are reorganized, among others (Driever and Nusslein-Volhard, 1988; Mahowald et al., 1983; Page and Orr-Weaver, 1996; Theurkauf et al., 1992). Thus activation must couple a developmental signal with control of the meiotic cell cycle. The mechanisms underlying activation remain unknown because this area has been largely unaddressed.

Several lines of reasoning suggest that new protein synthesis may be required for release from the metaphase I arrest and the completion of meiosis. Oocytes from Drosophila mothers mutant for *grauzone* and *cortex* arrest normally at metaphase I, and then when activated go through an aberrant anaphase I and arrest terminally in meiosis II (Page and Orr-Weaver, 1996). In addition to the meiotic arrest phenotype, these two genes appear to be required for the proper translation of BICOID protein after activation, and may also be required for polyadenylation of some messages (Lieberfarb, et al., 1996). One possible interpretation of these phenotypes is that *grauzone* and *cortex* are required for the translation of many messages, some of which are required for the completion of meiosis. This seems like a reasonable order of events since activation appears to be accompanied by a burst of translational activity in Drosophila oocytes (Mahowald et al., 1983). Finally, comparisons with the meiotic cell cycle in other animal oocytes suggest that the synthesis of new proteins may be required for the meiotic divisions.

In this study, we wanted to understand the role of new protein synthesis in the meiotic cell cycle of Drosophila oocytes. Unfortunately, it has been difficult to study the cytological events of meiosis in Drosophila females. As with some other organisms, the meiotic divisions generally take place inside the mother. However, in contrast to other model meiotic organisms, Drosophila females lay single eggs serially throughout their adult lives, rather than in seasonal spawnings. Thus, oocytes are activated to enter meiosis one at a time (for reviews, see Foe, et al., 1993; Spradling, 1993). Another confounding problem is that once an oocyte is activated, the meiotic divisions happen very quickly, and are complete within about 20 minutes (Riparbelli and Callaini, 1996); often they are completed before the egg is laid. The studies that have characterized the meiotic cytology of oocytes have been laborious because they have required ovary dissection or rapid collection of a few or single laid eggs (Dävring and Sunner, 1973; Huettner, 1924; Riparbelli and Callaini, 1996; Sonnenblick, 1950). Since most mutants produce eggs with reduced frequency, only healthy wild-type stocks are amenable to such studies, and thus there are few cytological descriptions of meiotic mutants beyond the metaphase I arrest. Because of these limitations, studies of the meiotic divisions in females have been limited to description, rather than experimental manipulation.

In order to study the meiotic divisions, we developed and present here a method for activating eggs in vitro, using a technique for isolating large quanti-

ties of oocytes developed by Theurkauf and Hawley (1992). We demonstrate that eggs activated in vitro go through meiosis normally, and we refine the stages of release from metaphase arrest, which were previously inaccessible to observation. We use this method to test the translational requirements for the progression of meiosis. We find that, contrary to our expectations, Drosophila oocytes do not need synthesis of new proteins to complete meiosis, nor to maintain the metaphase I arrest. Our in vitro system for activating eggs will enable the meiotic cell cycle to be more easily studied in Drosophila. In addition this system will be useful to researchers studying other aspects of meiosis by allowing genetic, cytological, and even biochemical studies to be performed in the same organism.

Materials and Methods

Egg Activation

Late-stage oocytes were isolated in a modification of the procedure of Theurkauf and Hawley (1992; also described in Theurkauf, 1994). Oocytes could be obtained from as few as 100 fattened females, although we had best results with 300 fattened females. Females were fed on wet yeast for 4-10 days, and then ground by pulsing 3-6 times at low speed in a blender in freshly made Isolation Buffer (IB: 55 mM NaOAc, 40 mM KOAc, 110 mM sucrose, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM HEPES; final pH 7.4 with NaOH; in some experiments, the pH was 8.1 and the final concentration was 94% that of full strength IB, with similar results.) The homogenate was filtered through a 650 µm mesh, and the material collected on the mesh was returned to the blender to repeat the procedure twice more, except that the final blending was a 10 - 15 second purée. The pooled homogenate was filtered through a 250 µm mesh to separate oocytes from larger body parts, and then oocytes were collected on a 125 µm mesh which filtered out smaller egg chambers. Filtration through the 250 µm mesh and collection on the 125 µm mesh were repeated, and then the oocytes went through 6-12 rounds of gravity settling in IB, with the supernatant removed and fresh IB added. This procedure was completed in exactly 15 minutes from the time of the first blender pulse, and resulted in populations highly enriched for unactivated stage 13 and 14 oocytes. An additional 10 minute incubation in IB was sometimes performed (see below).

Oocytes were activated according to a procedure modified from Mahowald et al (1983), in which oocytes in IB were washed in several changes of Activating Buffer (AB) for 5 minutes (3.3 mM NaH_2PO_4, 16.6 mM KH_2PO_4, 10 mM NaCl, 50 mM KCl, 5% PEG 8000, 2 mM $CaCl_2$, brought to pH 6.4 with 1:5 NaOH:KOH). The AB was then removed by washing in modified Zalokar's Buffer (ZAB), demonstrated by Limbourg and Zalokar to support growth of embryos with permeabilized vitelline membranes (Limbourg and Zalokar, 1973; ZAB: 9 mM $MgCl_2$, 10 mM $MgSO_4$, 2.9 mM NaH_2PO_4, 0.22 mM NaOAc, 5 mM glucose, 27 mM glutamic acid, 33 mM glycine, 2 mM malic acid, 7 mM $CaCl_2$, brought to pH 6.8 with 1:1 NaOH:KOH). Length of activation was measured as time after the first addition of AB.

Oocytes isolated in IB were not activated until the addition of AB. This was established in two ways. First, oocytes incubated in IB were destroyed by a 3

minute incubation in 50% bleach (data not shown), indicating that the vitelline membranes had not become crosslinked, as happens at egg activation (see Results section). Secondly, DNA staining of oocytes isolated and incubated in IB for various times up to 60 minutes revealed that such oocytes remained arrested at metaphase I (Table 3-2, Figure 3-7, and data not shown).

Drug Treatment and Metabolic Labeling

Cycloheximide (Fluka or Sigma) and chloramphenicol (Sigma) were used at a final concentration of 100 μg/ml. Increasing the cycloheximide concentration to 500 μg/ml did not improve translational inhibition as measured by metabolic labeling. Colchicine (Sigma) was used at a final concentration of 10 μg/ml. Drugs were added to the IB before the initial grinding of the flies and were included in all subsequent IB rinses. For activation experiments, cycloheximide and chloramphenicol were also added to the AB and ZAB. For metabolic labeling, oocytes that had been isolated in exactly 15 minutes as above were then incubated for exactly 10 minutes in fresh IB containing 0.1- 1 mCi/ml of ^{35}S-methionine (Amersham), and sometimes also containing appropriate inhibitors.

Fixation and Staining

Fixation was performed in one of three ways. To calculate efficiency of activation, activated eggs were fixed in their chorions by incubating in methanol. To select for activated eggs or to visualize meiotic figures, an aliquot of activated oocytes was taken at the appropriate time and dechorionated in 50% fresh Chlorox bleach for 3 minutes, then devitellinized and fixed by shaking in a two phase mixture of methanol/heptane. The mixture was removed after devitellinization, and replaced with fresh methanol. Eggs fixed in methanol were rehydrated by incubation in a PBS/methanol series before staining. Eggs activated less than about 40 minutes were often lost at the devitellinization step. Therefore, to view the early stages of meiosis, we dechorionated as above and then fixed for 10 minutes in 8% EM grade formaldehyde (Ted Pella) in a cacodylate buffer as described for oocyte fixation (Theurkauf, 1994). After several washes in PBS, eggs were rolled out of their vitelline membranes between two glass slides as described (Theurkauf, 1994). Eggs were extracted in 1% Triton for 1-2 hours before antibody labeling.

For spindle staining, eggs were incubated with a mouse monoclonal antibody against ß-tubulin (Amersham) at a concentration of 375 ng/ml in PBST (130

mM NaCl, 70 mM Na_2HPO_4, 35 mM NaH_2PO_4, 0.3% Triton X-100); or with two rat monoclonal antibodies against tubulin, YL1/2 and YOL1/34 (both from Sera-lab), each diluted 1:5 in PBST. The rat antibodies were better able to detect meiosis I spindles than the mouse antibodies. Mouse antibodies were detected by DTAF-labeled goat anti-mouse (Jackson), and rat antibodies were detected with either a DTAF-conjugated or a Texas Red-conjugated goat anti-rat antibody (both from Jackson). DNA was detected either by staining with 5μg/ml 7-AAD (Molecular Probes) for 30 minutes, followed by 2 quick washes; or by staining with OliGreen (Molecular Probes) diluted 1:5000 in PBS with 0.1% Triton and 20μg/ml of RNaseA for 30 minutes.

Microscopy

Samples were dehydrated in methanol and mounted on slides in clearing solution (2:1 benzyl benzoate: benzyl alcohol; Theurkauf and Hawley, 1992) containing 50 mg/ml n-propyl gallate to protect against photobleaching. Slides were scored with a Zeiss Axioskop fluorescence microscope equipped with 5X and 40X dry Plan Neofluar objectives. Experiments comparing activation in the presence and absence of cycloheximide were scored blind. All images were taken with a Bio-Rad MRC 600 confocal laser scanning head equipped with a krypton/argon laser, mounted on a Zeiss Axioskop microscope, with a 40X oil Plan Neofluar objective. In some cases, optical sections were taken and projected into a single plane. Images were processed on a Macintosh Power PC with the program Adobe Photoshop.

Translation Assays

Translation was assayed by isolating oocytes, incorporating ^{35}S-methionine, activating, bleaching, devitellinizing in methanol/heptane, fixing in methanol, and staining with 7-AAD, as above. Eggs that had completed meiosis were individually picked in an adaptation of the technique of Edgar et al (1994). Briefly, eggs were resuspended in clearing solution, and viewed without a cover-slip on a slide bounded by a corral of dried Elmer's Glue-All. We examined eggs under a fluorescence microscope for the presence of the four meiotic products, evidence of the completion of meiosis. We used surgical tweezers to remove eggs that had completed meiosis to an eppendorf tube of methanol, which dissolved the clearing solution. 5-20 eggs were collected for each sample, and these were rehydrated in PBS, then air dried and crushed with a melted Pasteur

pipet. For making extracts of labeled unactivated oocytes to assess translation during the metaphase I arrest, we picked oocytes that had lost all follicle and nurse cells, and that showed evidence of an elongated nucleus. A 1:1 mixture of EB:4x Laemli sample buffer was added (EB, described by Edgar, et al. [1994]: 10 mM Tris pH 7.5, 80 mM Naß-glycerophosphate pH 7.5, 20 mM EGTA, 15 mM $MgCl_2$, 2 mM Na_3VO_4, 1 mM benzamidine, 1 mM sodium meta-bisulfite, 0.2 mM PMSF) in a volume of 0.5-1 µl per egg. Lysates were boiled for 10 minutes, stored at -20°, and boiled for 10 minutes before loading. Samples were run on 14% or 16% 200:1 (acrylamide: bis) 0.75 mm gels, which were stained with Coomassie, washed in 40% methanol or water, and dried. Incorporated counts were quantitated with a Fuji Phosphoimager. Protein levels were quantitated by scanning the gels into computer files and analyzing with NIH Image software. By calibrating the program with known amounts of proteins, we ensured we were working within a range of linear response.

RESULTS

In Vitro Activation of Meiosis

The cytology of female meiosis in Drosophila has been characterized in painstaking studies of single or small numbers of oocytes dissected from the uterus or collected immediately after laying (Dävring and Sunner, 1973; Huettner, 1924; Riparbelli and Callaini, 1996; Sonnenblick, 1950). The mature oocyte arrests in metaphase of meiosis I, waiting to be activated during passage through the oviduct (Fig. 3-1A). Soon after activation, meiosis is resumed, whether or not the egg is fertilized (Doane, 1960). The oocyte passes quickly through the meiotic stages, and these are depicted in Figure 3-1 as observed in earlier studies. Cytokinesis does not occur in the meiotic divisions in Drosophila, and so polar bodies are not extruded. Rather, the oocyte retains the products of both meiotic divisions. After anaphase II, the four telophase nuclei, which are arranged in a line determined by the orientation of the meiosis II spindles, decondense and appear to be in interphase (Fig. 3-1D). In an unfertilized egg the interphase nuclei migrate together, recondense their chromatin (Fig. 3-1E), and then fuse to form one or two rosette structures. The fused polar bodies have been likened to rosettes because individual arms are circularly arranged with their centromeres on the inside (Fig. 3-1F). In fertilized eggs, three of the four meiotic products fuse to form the rosette, while the fourth joins the male pronucleus to form the zygotic nuclei.

We were interested in efficiently activating oocytes in vitro for use as a tool for studying meiosis in Drosophila. We began our efforts by building on the pioneering work of Mahowald et al (1983), who investigated a number of conditions for activating mature oocytes dissected out of females. Rather than dissecting, we took advantage of a blender method developed by Theurkauf (1994) that allowed us to harvest many hundreds of oocytes to use as our starting material (Fig. 3-1A). Mahowald et al. did not examine meiotic cytology in detail (1983), but in our hands, their optimized conditions did not cause eggs to complete meiosis normally. Instead, we worked out conditions where we activated eggs in the Mahowald buffer for a pulse, followed by incubation in a physiological buffer (see materials and methods).

Eggs activated in this manner progressed through a cytologically normal meiosis (Fig. 3-2). We fixed activated eggs in either methanol or formaldehyde and stained them with anti-tubulin antibodies and a DNA stain to observe the

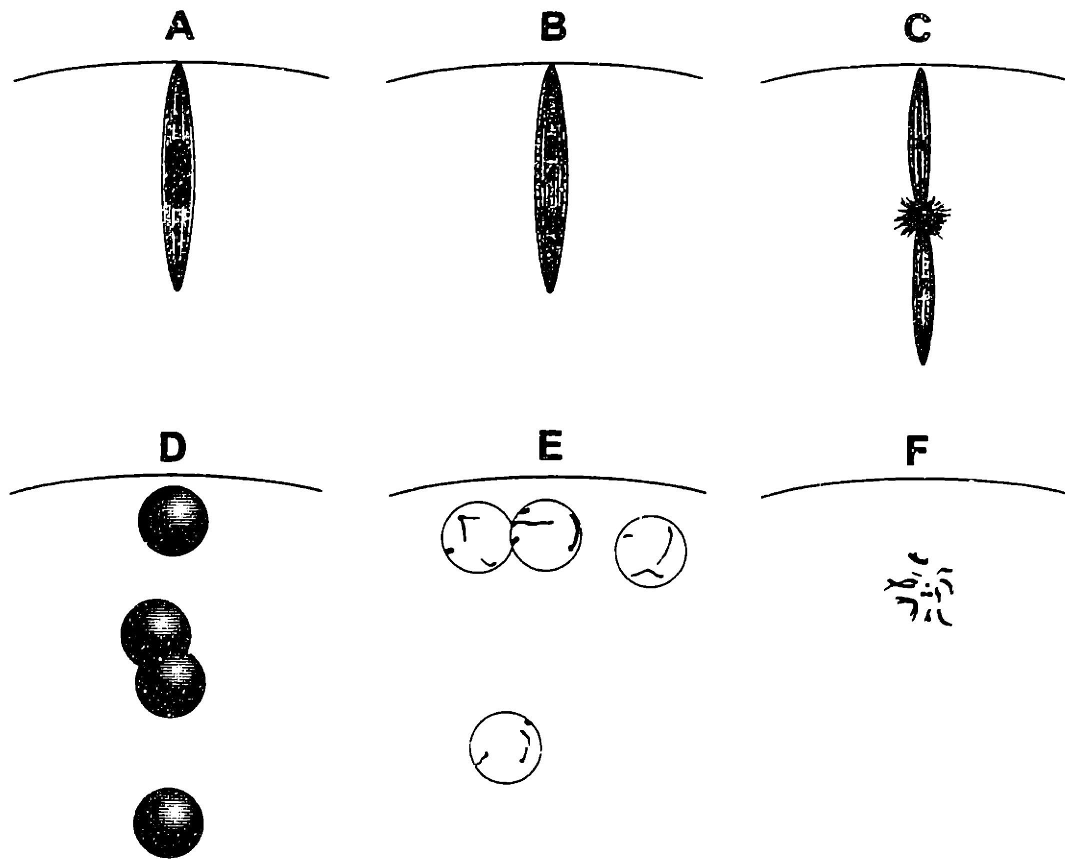


Figure 3-1. The stages of meiosis in oocytes activated in vivo as they have been previously characterized.
Stages are based on oocytes dissected from the oviduct before deposition, and eggs collected immediately after deposition. A is adapted from Theurkauf and Hawley (1992); B -F are adapted from Huettner (1924).

A) Spindle and chromosomes of a mature stage 14 metaphase-I arrested oocyte. The DNA is elongated along the axis of the spindle, and the tiny fourth chromosomes are sometimes observed to have precociously migrated poleward.
B) Spindle and chromosomes of an oocyte in anaphase I.
C) Two spindles with chromosomes in a metaphase II oocyte. Recent studies have identified an aster-like midbody between the spindles (Riparbelli and Callaini, 1996).
D) The four meiotic products after the completion of meiosis. The chromatin decondenses to give an interphase-like appearance, and the nuclear envelope appears intact. In an unfertilized egg, all four meiotic products begin to migrate together at this stage.
E) Chromosomes beginning to recondense inside the nuclear envelope of the four meiotic products.
F) The rosette structure. In an unfertilized egg, the condensed chromatin of the four meiotic products fuse to form this structure, whereas in a fertilized egg, only the three unused meiotic products form a rosette.

Figure 3-2. The stages of meiosis are normal in oocytes activated in vitro.

Oocytes were isolated and activated as described, and the sequential stages of meiosis are shown. The cytology is similar to that sketched in figure 1. DNA is represented in red and tubulin in green. Scale bars are approximately 5 µm.

A) The metaphase I arrested stage 14 oocyte. The DNA is elongated along the axis of the spindle, and the tiny fourth chromosomes are separated from the mass of chromatin (arrows). Individual chromosomes are not visible in the chromatin mass.

B) Early anaphase I. The first step for resuming meiosis appears to be the individuation of chromosomes.

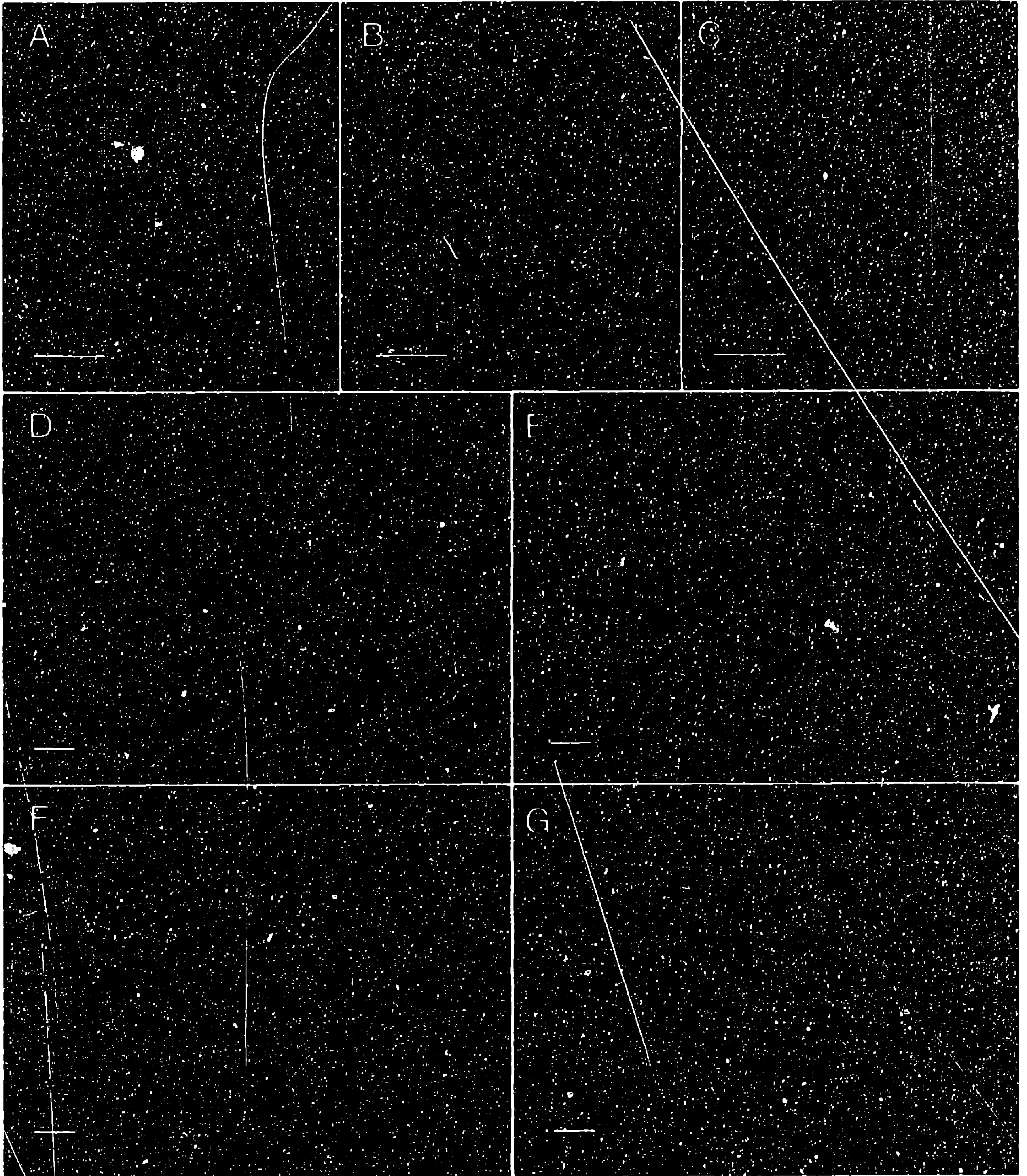
C) Late anaphase I.

D) Anaphase II. The midbody is often visible between the two spindles.

E) The four meiotic products with decondensed chromatin.

F) The meiotic products recondensing their chromatin. Only three of the four are visible in this image.

G) The rosette structure containing the fused condensed meiotic products. The rosette structure has tubulin at its center, as is observed in laid eggs (Page and Orr-Weaver, 1996).


A
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stages of meiosis. Eggs undergoing meiotic divisions were clearly identified by the presence of a meiosis I or two meiosis II spindles (Fig. 3-2B,C,D). Although other researchers have been able to observe some meiosis I events by activating with hypotonic buffers, the stages after meiosis I have been elusive (Hatsumi and Endow, 1992; Puro and Nokkala, 1977). With our method, eggs continued into meiosis II (Fig. 3-2D), the post-meiotic interphase (Fig. 3-2E), and the recondensation of chromatin to form the rosette structures (Fig 3-2F,G). We were able to activate up to several hundred oocytes at once.

By activating eggs in vitro, we were able to compare directly the metaphase I and anaphase I chromosomes. We noted that the first step in resuming meiosis appeared to be the individuation of chromosome arms. In metaphase-I arrested oocytes, a large mass of chromatin is observed at the metaphase plate, and often the tiny fourth chromosomes are observed between the poles and the metaphase plate (Fig. 3-2A). In the chromatin mass at the plate, we observed no chromosome arms, individual chromosomes, or visible structure of any kind by conventional or confocal microscopy. These observations agree with those of other researchers (Theurkauf and Hawley, 1992). However, once oocytes were activated, individual chromosome arms became visible, possibly indicating an increase in chromatin condensation (Fig. 3-2B; Fig 3-3). These morphological changes occurred before poleward movement was observed. We believe this represents a significant change in chromosome structure between metaphase-arrested and anaphase I chromosomes.

Another effect of egg activation is a change in the vitelline membrane which lies just under the chorion. Before activation, the vitelline membrane is permeable to many small molecules, but after activation the membrane becomes cross-linked and impermeable (Ashburner, 1989; Spradling, 1993). Mahowald et al. demonstrated that bleach could be used as a quick selection for activated oocytes, since bleach dissolves the entire unactivated oocyte, whereas bleach dissolves only the outer chorion of activated eggs (1983). Eggs activated by our method also become impermeable to bleach within a few minutes (data not shown).

Estimates of the efficiency of activation are hampered because we used the rapid blender method of isolating oocytes. Although this method is much faster than dissection, the drawback is that a variable percentage of the isolated oocytes are in earlier stages of development and cannot be activated, in addition to the desired mature stage 14 oocytes. For most experiments, we chose to destroy

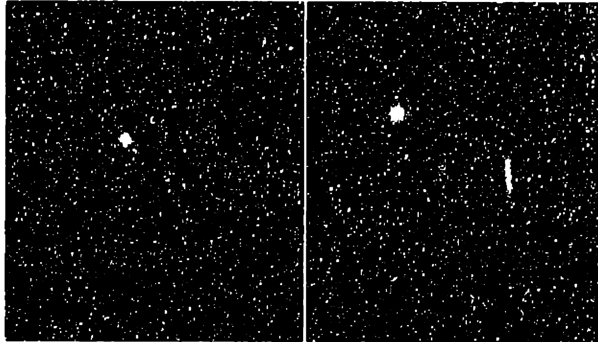


Figure 3-3. Chromosomes become individuated after activation and before beginning poleward movement. Eggs were fixed in formaldehyde and stained to visualize DNA.

immature oocytes by the bleach treatment which selects for activated eggs. To estimate activation efficiency, however, we omitted the bleach treatment, thus including immature oocytes in our "unactivated" oocyte percentage. Eggs were activated for 25 minutes, fixed directly in methanol, stained with a DNA stain, and examined for meiotic stage. Examining over a hundred oocytes, we found that 60-70% of the heterogeneous starting material was activated, and over half of those had completed the meiotic divisions. This suggests that most of the metaphase-arrested stage 14 oocytes were activated by our method.

We found the timing of the meiotic divisions in eggs activated in vitro to be comparable to that of eggs activated in vivo. After 25 minutes, 22% of the in vitro activated eggs were in meiosis II, and another 71% had finished both meiotic divisions. After 40 minutes, over 90% of the eggs had completed the meiotic divisions (Table 3-1 and Fig. 3-4A). Studies on the timing of the meiotic divisions in laid eggs have reported that 20 minutes after egg deposition, 66% of eggs were in meiosis II, and the remaining 34% had progressed further (Riparbelli and Callaini, 1996). These numbers demonstrate that eggs activated in vitro progress through meiosis at a rate similar to that of laid eggs.

After the divisions, the eggs activated in vitro passed through a cytologically normally post-meiotic interphase (Fig. 3-2E), and in many of them the chromatin of the meiotic products recondensed and fused to form rosette structures (Fig. 3-2F,G). Instead of arresting with rosette structures as would an unfertilized laid egg, however, the nuclei of eggs activated in vitro often continued to replicate and divide in aberrant ways. Additionally, the timing of events became delayed sometime around the stage of recondensation. In laid fertilized eggs, full recondensation of the meiotic products happens synchronously with the first mitosis, only about 10 minutes after the completion of meiosis, whereas in these experimental eggs, rosette structures were much slower to form (Table 3-1 and Fig. 3-4A). This timing and behavior suggests that our in vitro activation method is useful for studying events through the post-meiotic interphase, but not further.

Is Protein Synthesis Required for the Completion of Meiosis?

We wanted to understand how the resumption of meiosis was regulated at activation. It has been demonstrated that at activation ribosomes from arrested Drosophila oocytes are recruited into polysomes, suggesting that the rate of new protein synthesis is significantly enhanced (Mahowald et al., 1983). Additionally,

Table 3-1.
The stages of meiosis observed at different times after in vitro activation

time after activation[a] (min.)	cycloheximide present?	meiosis I (see Fig. 3-2A,B,C)	meiosis II (see Figs. 3-2D, 3-5A)	3-4 normal meiotic products[b] (see Figs. 3-2E, 3-5B)	3-4 huge meiotic products[b] (see Fig. 3-5C)	recondensing (see Fig. 3-2F)	rosette/ mitotic divisions (see Fig. 3-2G)	n
25	no	7%	22%	53%	0	13%	5%	45
40	no	1%	7%	24%	12%	38%	18%	92
60	no	1%	0	12%	5%	40%	41%	92
90	no	2%	1%	1%	0	12%	84%	98
120	no	0	1%	4%	0	7%	88%	83
25	yes	16%	23%	51%	5%	2%	2%	92
40	yes	3%	9%	21%	54%	9%	3%	94
60	yes	3%	2%	5%	71%	18%	0	92
90	yes	1%	1%	2%	13%	81%	1%	97
120	yes	1%	1%	0	10%	88%	0	94

[a]At the appropriate time after activation, activated eggs were selected with bleach, devitellinized, fixed in methanol, and immunostained with anti-tubulin and a DNA stain.

[b]It was not always possible to see the fourth meiotic product when it was located in the interior of the egg.

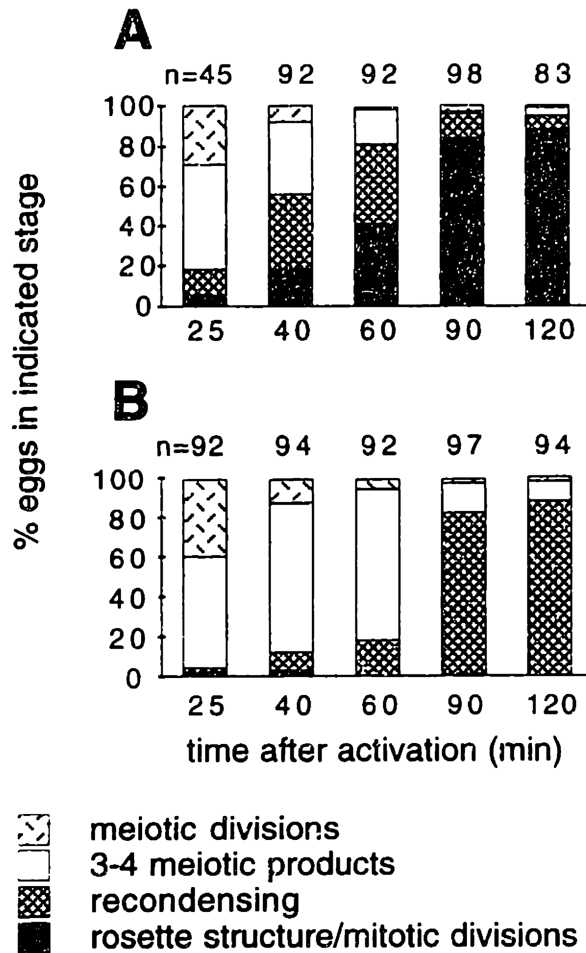


Figure 3-4. A graphic representation of the stages of meiosis observed in eggs activated in vitro.
This data is presented numerically in Table 3-1.

A) Activated eggs.

B) Eggs activated in the presence of cycloheximide.

it is known that some proteins, such as BICOID and STRING, are not present in the metaphase-arrested oocyte and are selectively translated only after activation (Driever and Nusslein-Volhard, 1988; Bruce Edgar, personal communication). In Xenopus, mice, clams, and starfish, treating oocytes with translational inhibitors perturbs or inhibits the meiotic divisions, indicating that in many species translation is required for the resumption and/or progression of meiosis (Clarke and Masui, 1983; Fulka Jr. et al., 1994; Galas et al., 1993; Gerhart et al., 1984; Hunt et al., 1992; Kanki and Donoghue, 1991; Picard et al., 1985). We suspected that the burst of protein synthesis accompanying Drosophila egg activation was required for the proper execution of meiosis, in part because of comparisons with other organisms, and in part because the mutations *grauzone* and *cortex*, which arrest meiosis at metaphase II, also display defects in translation (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996) We tested this hypothesis by activating eggs in the presence of the protein synthesis inhibitor cycloheximide, and examining whether they could complete meiosis.

We blocked protein synthesis by adding 100 μg/ml cycloheximide to all buffers used before fixation. Oocytes remain unactivated during the mass isolation step (see Materials and Methods), and so by including cycloheximide in the Isolation Buffer we were able to inhibit protein synthesis before egg activation and the resumption of meiosis. Since the vitelline membrane is permeable to many solutes before activation, we expected that cycloheximide would be able to enter the oocyte (see below for confirmation). Oocytes were activated in the presence of the inhibitor, selected with bleach, fixed in methanol, and stained with anti-tubulin antibodies and a DNA stain. Strikingly, eggs progressed through both meiotic divisions normally. In 25 minute activation experiments, we observed many eggs with normal meiosis II spindles, some with a pronounced mid-body as has been previously observed in eggs activated in vivo (Fig. 3-5A; Riparbelli and Callaini, 1996) We also observed many eggs with four condensed collinear nuclei and no spindles, indicative of telophase II (data not shown). After the meiotic divisions, the four meiotic products began to decondense normally for the post-meiotic interphase (Fig. 3-5B).

The timing of the meiotic divisions was similar between cycloheximide and untreated activated eggs: after 25 minutes, 39% of the cycloheximide-treated eggs were in the meiotic divisions, compared to about 29% of control eggs (Table 3-1 and Fig. 3-4); and for both cycloheximide and drug treated eggs, about half of them were in the post-meiotic interphase after 25 minutes. Interestingly,

Figure 3-5. Eggs treated with cycloheximide can complete the meiotic divisions and decondense their chromatin normally.

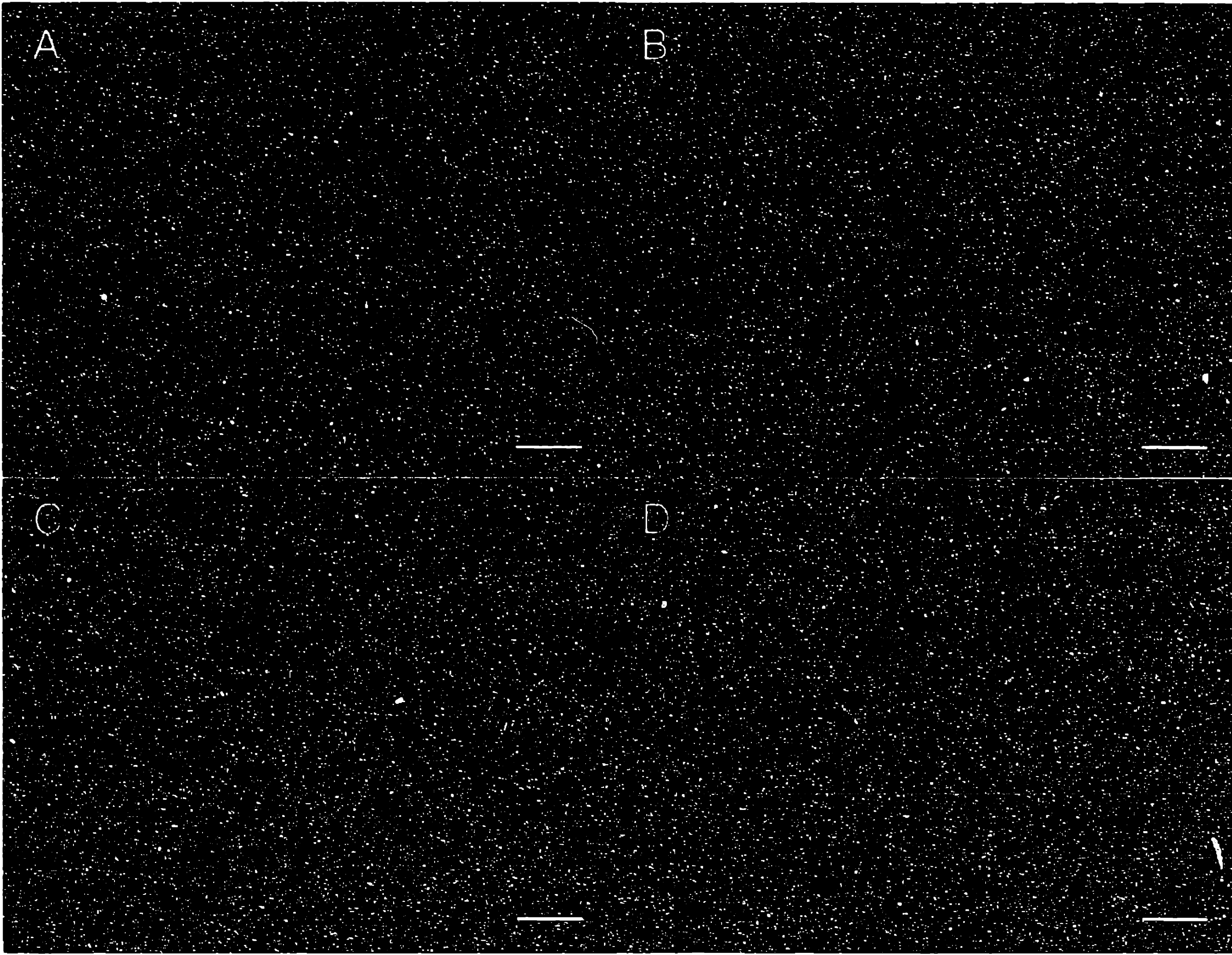
Late stage oocytes were treated with 100 μg/ml cycloheximide for 25 minutes before activation, and then activated and incubated in the continuing presence of cycloheximide. The sequence of events after activation is shown. DNA is represented in red and tubulin in green. Scale bars are approximately 20 μm.

A) Meiosis II proceeds normally in cycloheximide-treated eggs. The inner spindle is faintly stained in this image, and the midbody is visible between the spindles.

B) The four meiotic products after meiosis. Chromatin appears to decondense normally in the continuing presence of cycloheximide.

C) Enlarged meiotic products. The DNA does not appear to be overreplicating, since the staining is very faint in the large cleared areas.

D) Abnormal recondensation of the chromatin. Chromosomes recondense separately rather than together, and the nuclear envelope appears to retain its integrity, as visualized by the continued presence of the cleared area.


A
B
C
D

decondensation progressed to form abnormally large nuclei that were visible as large areas of clearing in the cytoplasm (Fig. 3-5C). After 90 minutes or so, the DNA recondensed within these large nuclei, but the large cleared areas persisted, indicating the nuclear envelope did not break down (Fig. 3-5D; Table 3-1). Thus, it appeared that new protein synthesis is not required for the completion of meiosis in Drosophila females, although it is required for proper recondensation of the DNA after the post-meiotic interphase.

In order to ensure that cycloheximide had entered the oocyte and blocked protein synthesis, we metabolically labeled oocytes with ^{35}S-methionine and then examined incorporation of the label into total protein extracts. Eggs were isolated in buffer with or without cycloheximide, labeled, activated, then fixed in methanol, stained, and examined under a fluorescence microscope. Eggs that had completed meiosis, as judged by the presence of four meiotic products, were individually picked to make protein extracts. Eggs that were activated in the presence of cycloheximide incorporated about 3-5% of the label that the untreated controls incorporated in repeated experiments (Fig. 3-6). This confirmed that both cycloheximide and methionine could enter the unactivated oocyte through the vitelline membrane. Cytological examination of unactivated eggs that were drug-treated and labeled confirmed that the labeling procedure did not activate meiosis prematurely (data not shown).

Since the speed of meiosis remained relatively constant even with protein synthesis reduced to 5% of normal levels, it seemed likely that no new proteins were required for the completion of meiosis. However, we were unable to rule out the possibility that a putative meiotic "activating protein" was translated in that 5% of synthesis. Since increasing the concentration of cycloheximide five-fold did not decrease the total protein synthesis (data not shown), we considered the possibility that the remaining cycloheximide-resistant protein synthesis was mitochondrial. Such synthesis would be inhibited by prokaryotic translation inhibitors like chloramphenicol, but not by eukaryotic translation inhibitors such as cycloheximide. When eggs were incubated in a combination of cycloheximide and chloramphenicol, labeled, activated, fixed, stained, and hand-picked to make protein extracts, we found that the level of incorporation of ^{35}S-methionine was reduced to about 1% of control extracts in multiple experiments (Fig. 3-6). The most credible explanation is that no new protein(s) are required for the completion of meiosis after the metaphase arrest in Drosophila females. A protein not

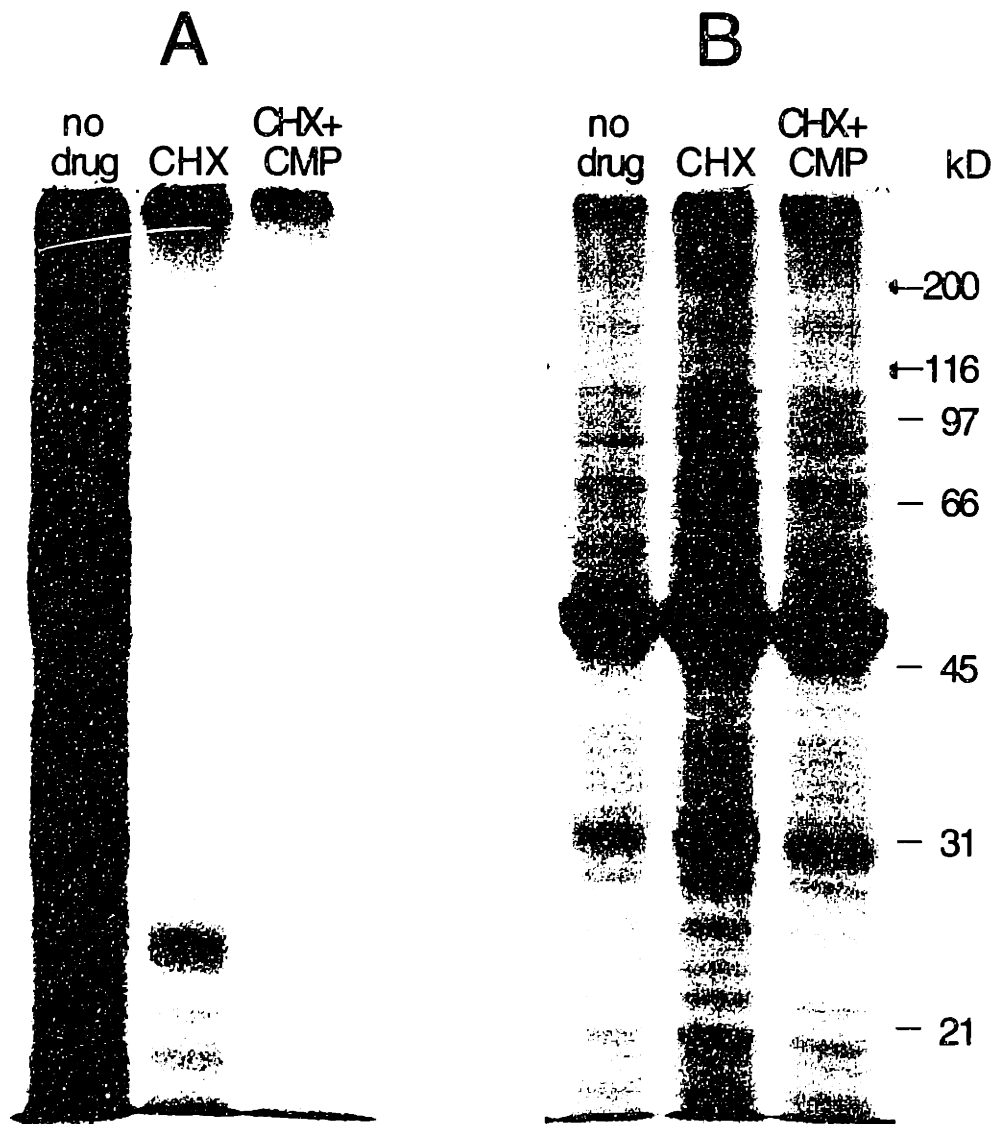


Figure 3-6. Protein synthesis is inhibited in eggs treated with cycloheximide.

Oocytes were incubated in the absence or presence of inhibitor(s) for 15 minutes, and then incubated in ^{35}S-methionine in the absence or presence of inhibitor(s) for 10 minutes. Following incubation, oocytes were activated as described. After 25 minutes, activated eggs were selected with bleach, devitellinized, fixed in methanol, and stained for DNA. Protein extracts were made from eggs that were observed by fluorescence microscopy to have completed meiosis, as judged by the presence of four meiotic products. CHX= 100 µg/ml cycloheximide; CHX+CMP= 100 µg/ml each of cycloheximide and chloramphenicol.
A) Digital image of radioactivity incorporated into activated eggs. Eggs activated without drugs incorporate ^{35}S-methionine at 100%, whereas in the experiment shown here eggs activated in the presence of cycloheximide incorporate 3.1%, and eggs activated in the presence of cycloheximide and chloramphenicol incorporate 1.5%.
B) The same gel shown in A, stained with Coomassie to show all proteins.
% incorporation was determined by normalizing for protein loading. Molecular weight markers are shown at the right.

present in the unactivated oocyte is clearly required, however, for proper recondensation of the DNA after meiosis is completed.

The Metaphase I Arrest Is Maintained in the Absence of Protein Synthesis

In repeated experiments, we found that variable but measurable protein synthesis occurred in the metaphase-I arrested oocytes (data not shown). This variability may correlate with the observation that the longer an oocyte is arrested in metaphase I, the fewer of its ribosomes are incorporated into polysomes (Mahowald et al., 1983). Although we determined that protein synthesis was not required for the regulation of meiosis after the metaphase I arrest, it was still possible that the continued synthesis of new protein(s) was required to maintain the metaphase I arrest. Indeed, in the marine mollusk Patella, maintenance of the normal arrest at metaphase I requires continuing synthesis of cyclins A and B. If translation is inhibited, or if those messages are inactivated, the Patella oocyte nucleus returns to an interphase state without passing through meiosis I anaphase, in effect going backwards through the meiotic cell cycle (Néant and Guerrier, 1988; van Loon et al., 1991).

To test the possibility that continued synthesis is required to maintain the metaphase I arrest in Drosophila, we incubated unactivated oocytes in control or cycloheximide-containing media for 30 or 60 minutes, fixed and stained them, and examined them for nuclear morphology. Nuclear morphology has been demonstrated to be a good indicator of the stage of meiosis after prophase I, since prophase and prometaphase nuclei appear round, whereas metaphase-I arrested nuclei appear elongated along the pole-to-pole axis of the spindle (Theurkauf and Hawley, 1992; Fig. 3-2A), probably because of the tension from the spindle pulling on attached homologs; anaphase I and meiosis II can also be assessed from nuclear morphology. After 60 minutes of incubation in control media, oocytes did not proceed into anaphase I and remained arrested at metaphase I (Fig. 3-7A), although it appeared that the nuclear elongation increased with the length of the arrest (data not shown). We found that 67% of oocytes incubated for an hour in control media had elongated nuclei typical of metaphase (Table 3-2). In late oocyte development, the stage of meiosis is not exactly correlated with the developmental stage of oocyte growth; hence the 33% of nuclei that appeared round were probably in prophase or prometaphase. We observed the same morphology in oocytes incubated in cycloheximide with roughly the same frequency (Fig. 3-7B; Table 3-2), demonstrating that new proteins, such as cyclins, are not required for

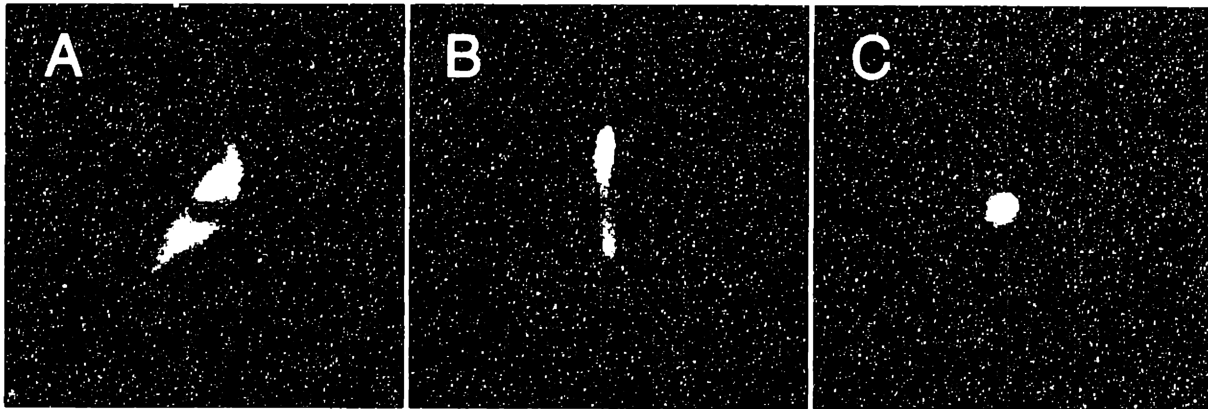


Figure 3-7. Oocytes treated with cycloheximide maintain the metaphase-I arrest.

Oocytes were incubated in the presence or absence of inhibitor for 60 minutes, fixed in methanol, stained for DNA, and observed by fluorescence microscopy.

A) Oocytes incubated without inhibitors maintain the metaphase I arrest, as indicated by the elongation of the chromatin.

B) Oocytes incubated with 100 μg/ml cycloheximide also maintain the metaphase I arrest, as indicated by the elongated chromatin.

C) Oocytes incubated with 10 μg/ml colchicine, a microtubule depolymerizing drug, are round and not elongated, demonstrating that depolymerization of the metaphase I spindle causes a change in chromatin conformation.

Table 3-2.
Cycloheximide has no visible effect on metaphase arrested nuclei

	round nucleus (prophase/ no spindle)	elongated nucleus (metaphase-arrested)	two nuclei (anaphase or meiosis II)	n
untreated oocytes	33%	67%	0	55
cycloheximide-treated oocytes	28%	72%	0	71
colchicine-treated oocytes	100%	0	0	25

the maintenance of the metaphase I spindle. In both the drug-treated and untreated oocytes, the chromosomes remained indistinct and amorphous during the prolonged arrest. As a positive control, to ensure that we could detect the breakdown of the metaphase I spindle by nuclear morphology, we also incubated oocytes in the microtubule-depolymerizing drug colchicine for the same amount of time. The rounding of the oocyte nucleus in colchicine was clearly different from the elongated nucleus observed in cycloheximide-treated and control oocytes (Fig. 3-7C; Table 3-2). Thus, the continual synthesis of new proteins is not necessary for the maintenance of the metaphase I arrest of Drosophila female meiosis.

Discussion

We have developed an in vitro system for activating Drosophila oocytes, and have demonstrated that oocytes activated in this manner proceed through cytologically normal meiotic divisions. Our method can activate hundreds of oocytes at once, and they progress accurately through meiosis at approximately the same speed as oocytes activated in vivo. This system will be useful for investigators analyzing the effect of mutations on meiosis, or for studying the localization of a known protein during meiosis. It will also be useful for assessing the stability and forms of proteins during the meiotic cell cycle.

An unexpected benefit of in vitro activation is that we were able to examine the transition out of the metaphase I arrest, a previously inaccessible transition in Drosophila. Studies have shown that in metaphase I arrested oocytes, the chromosomes are amorphous and not individuated, with no visible chromosome arms (Theurkauf and Hawley, 1992). Although other researchers have demonstrated that anaphase I and meiosis II chromosomes have condensed arms (Huettner, 1924; Riparbelli and Callaini, 1996), it has been unclear if the cytological differences observed between amorphous and condensed chromosomes were caused by differences in imaging methods. Using consistent fixation and imaging techniques, we are able to observe both that metaphase I chromosomes have little structure, and that immediately after activation, they become individuated. This change in chromosome structure, which may represent an increase in condensation, appears before the chromosomes have begun to travel poleward in anaphase I, and so it is not an artifact of poleward movement.

The observation that chromosome individuation is an early step in egg activation has ramifications for studies on the maintenance of the metaphase I arrest. It has been demonstrated that to maintain this arrest, at least one meiotic crossover event is required to link homologous kinetochores. In mutant oocytes without any such crossovers, Hawley and colleagues have shown that the metaphase I arrest is not maintained, and the nuclei progress into anaphase I and sometimes meiosis II without being activated. Interestingly, in these unactivated but not-arrested oocytes, the chromosomes remain amorphous and never form visible chromosome arms throughout anaphase I and meiosis II (Jang et al., 1995; McKim et al., 1993). This lack of chromosome structure implies that the normal pathway for the resumption of meiosis is not controlled by only the chromo-

somes. Other activation events besides the loss of attachment between homologs are required for the normal resumption of meiotic progression.

As a means of studying the role of translational regulation in the meiotic cell cycle, we demonstrated that small molecules including specific inhibitors can enter the oocyte before activation. Because they can be added at a precise time, the effects of inhibitors in the in vitro system are more easily interpreted than the previous method of feeding inhibitors to the fly. The radioactive labeling technique that we described here is useful not only for assessing the efficiency of translation; it can also be adapted to label oocyte and meiotic proteins for biochemical assays, such as immunoprecipitations.

The goal of this study was to analyze the role of protein synthesis in regulating the progression of meiosis. By inhibiting new protein synthesis during the metaphase I arrest, we found that in vitro activated eggs could complete meiosis without new protein synthesis. Thus, all the components necessary for the two divisions and the post-meiotic interphase are synthesized before the arrest. Proteins required for the suppression of DNA synthesis between the meiotic divisions must also be present in the metaphase-I arrested oocyte, acting as functional counterparts to Mos and cdc2 in other organisms (see Furuno, et al., 1994; Picard, et al., 1996). Studies of ribosomes in Drosophila suggest that at egg activation there is an overall increase in the rate of protein synthesis (Mahowald et al., 1983) but our results show that any newly translated proteins are not required during meiosis.

Recessive mutations in the Drosophila genes *grauzone* and *cortex* cause females to lay eggs that arrest abnormally at metaphase II of meiosis (Page and Orr-Weaver, 1996). These eggs also display defects in the polyadenylation of some messages, including *BICOID*, which appears not to be translated in these eggs (Lieberfarb et al., 1996). The aberrant meiotic arrest in *grauzone* and *cortex* eggs may be an indirect consequence of a generalized failure in translation. However, we show here that no protein synthesis is required after the metaphase I arrest for the completion of meiosis, and so the only way that a failure in translation could be responsible for the meiotic arrest phenotype is if the putative meiotic activator were translated earlier in oogenesis. Although there may be some evidence for an early defect in polyadenylation in *cortex* eggs (Lieberfarb et al., 1996), *grauzone* eggs appear normal until the time of egg activation. Thus egg activation itself may be the primary defect in *grauzone* eggs.

In apparent contrast to our results with Drosophila, inhibition of protein synthesis disturbs the meiotic divisions in oocytes of Xenopus, mice, clams, and starfish, demonstrating that translation plays an important role in the regulation of meiosis in these species (Clarke and Masui, 1983; Fulka Jr. et al., 1994; Galas et al., 1993; Gerhart et al., 1984; Hunt et al., 1992; Kanki and Donoghue, 1991; Picard et al., 1985). In mice, the addition of a translational inhibitor to oocytes at the start of maturation (the progression from prophase I arrest to metaphase II arrest) blocks meiosis I; and adding translational inhibitors in metaphase I blocks meiosis II (Clarke and Masui, 1983, and references therein). In Xenopus, the meiotic divisions have been staged in terms of MPF activity, which was believed to be high at metaphase I and metaphase II, and to dip between the divisions (Gerhart et al., 1984). The addition of cycloheximide to Xenopus oocytes at maturation blocks meiosis I, and the addition of cycloheximide before the second appearance of MPF blocks the second meiotic division (Gerhart et al., 1984). At both these points, synthesis of the Mos protein has been shown to be necessary but not sufficient for proper meiotic progression (Furuno et al., 1994; Kanki and Donoghue, 1991). However, inhibiting protein synthesis after MPF activity has begun to rise again has no effect on the progression of meiosis II (Gerhart et al., 1984). How these results in Xenopus compare to ours is unclear, because there is currently some confusion about how the oscillating levels of MPF are related to the meiotic divisions (Furuno et al., 1994; Ohsumi et al., 1994). It may be that in Xenopus new protein synthesis is required until some time in meiosis I, in which case the Drosophila cell cycle may be subject to similar translational regulation.

Experiments with translational inhibitors in clams and starfish have demonstrated the existence of a "commitment point" before the first meiotic cleavage that governs entry into meiosis II. Protein synthesis is required before the commitment point, but not after, in order for the oocytes to emit a second polar body (Hunt et al., 1992; Picard et al., 1985). Thus the meiotic divisions of clams, starfish, and Xenopus oocytes may all require protein synthesis up to some point in meiosis I. This may be compatible with our results in Drosophila: if a meiosis II commitment point exists in Drosophila, then it must occur before the metaphase I arrest. Yet Drosophila oocytes can also maintain and release a meiotic arrest without protein synthesis, whereas none of these other model organisms demonstrate that level of post-translational regulation.

We also tested whether the maintenance of the meiotic arrest at metaphase I required a continuing supply of new proteins. Since inhibiting protein synthe-

sis does not affect the cytology of the metaphase I arrest in Drosophila oocytes, we conclude that the metaphase arrest is a stable state requiring no continuing synthesis. In contrast, protein synthesis is required to maintain the meiotic arrest in oocytes of the marine mollusk Patella, which arrest at metaphase I, like Drosophila; and in mouse oocytes, which arrest at metaphase II. In both of these cases, it appears that the application of cycloheximide causes a decline in MPF activity, and the nuclei change to an interphase appearance (Clarke and Masui, 1983; Fulka Jr. et al., 1994; Moos et al., 1996; Néant and Guerrier, 1988). For Patella oocytes, two of the proteins required to maintain the arrest are cyclins A and B, as ablating these messages during metaphase I leads to the same decondensed nuclear phenotype as a 40 minute treatment with cycloheximide (van Loon et al., 1991). Drosophila metaphase-I arrested nuclei, in contrast, maintain a metaphase arrest after an hour of exposure to cycloheximide. It will be interesting to see whether Drosophila metaphase I oocytes arrest with high levels of MPF, as do metaphase I Patella oocytes and metaphase II mouse oocytes.

We have demonstrated that protein synthesis is required to recondense the chromatin after decondensation of the meiotic products at the end of meiosis II. Our results correlate with those of earlier studies on the effects of protein synthesis inhibitors in the mitotic divisions of the Drosophila embryo (Edgar and Schubiger, 1986; Zalokar and Erk, 1976). They found that inhibitor-treated nuclei could not enter mitosis, and arrested after S-phase with expanded nuclei similar to those we see in cycloheximide-treated oocytes. The similarity of morphology suggests that the same protein(s) may be required for the proper condensation of chromatin at the end of meiosis and before embryonic mitoses. This new protein is unlikely to be cyclin A or B, because in Drosophila embryos oscillation of the levels of cdc2/cyclin complexes is not detected in the early cleavage divisions, suggesting that synthesis of cyclins is not required before each mitosis (Edgar et al., 1994).

In summary, the metaphase-I arrested Drosophila oocyte contains within it all the proteins necessary to accomplish an astonishing variety of cell cycle events: maintaining a developmental arrest, resuming the cell cycle in response to an external signal, individuating chromosome arms, segregating homologs at anaphase I, repressing DNA synthesis between the divisions, segregating sister chromatids at anaphase II, decondensing the chromatin of the four meiotic products, and moving those meiotic products together. Protein(s) not available in the oocyte are first required to recondense the chromatin, a process that

normally occurs at the first mitosis in fertilized eggs. The system for activating eggs in vitro, in combination with genetic analysis, will be instrumental for further analysis of the meiotic cell cycle in Drosophila.

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Chapter Four
The Cohesion Protein MEI-S332 Localizes to Condensed Meiotic and Mitotic Centromeres until Sister Chromatids Separate

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† AWP localized MEI-S332-GFP throughout female meiosis, prepared the cycloheximide and untreated extracts for determining degradation after anaphase II, and localized MEI-S332-GFP in mitosis and on polar bodies.

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ABSTRACT

The Drosophila MEI-S332 protein has been shown to be required for the maintenance of sister-chromatid cohesion in male and female meiosis. The protein localizes to the centromeres during male meiosis when the sister chromatids are attached, and it is no longer detectable after they separate. *Drosophila melanogaster* male meiosis is atypical in several respects, making it important to define MEI-S332 behavior during female meiosis, which better typifies meiosis in eukaryotes. We find that MEI-S332 localizes to the centromeres of prometaphase I chromosomes in oocytes, remaining there until it is delocalized at anaphase II. By using oocytes we were able to obtain sufficient material to investigate the fate of MEI-S332 after the metaphase II/ anaphase II transition. The levels of MEI-S332 protein are unchanged after the completion of meiosis, even when translation is blocked, suggesting that the protein dissociates from the centromeres but is not degraded at the onset of anaphase II. Unexpectedly, MEI-S332 is present during embryogenesis, localizes onto the centromeres of mitotic chromosomes, and is delocalized from anaphase chromosomes. Thus, MEI-S332 associates with the centromeres of both meiotic and mitotic chromosomes and dissociates from them at anaphase.

INTRODUCTION

Cohesion between sister chromatids is essential for proper segregation of chromosomes during mitosis and meiosis. By counteracting spindle forces pulling chromosomes towards the poles, cohesive forces between sister chromatids enable stable bipolar attachments to be established; these in turn allow the sister chromatids to be partitioned appropriately during anaphase. The consequences of inappropriate partitioning can be severe: aneuploidy is observed in many tumors and also in individuals with congenital disorders such as Down syndrome. Defects in sister-chromatid cohesion have been suggested as an important factor that might be involved in oncogenesis or meiotic errors (Lamb et al., 1996; Lengauer et al., 1997; Orr-Weaver, 1996).

In both meiosis and mitosis, cohesion exists between the arms and the centromere regions of the sister chromatids after their replication, but release of sister-chromatid cohesion occurs differently in these two types of cell division (Moore and Orr-Weaver, 1998) In mitosis, the sister chromatids segregate from one another in a single cell division, and thus cohesion is released from both the chromosome arms and centromere regions at the same time, the onset of anaphase. Meiosis consists of two cell divisions that follow a single round of replication: the homologs segregate from one another in the first division, the sister chromatids in the second division. The homologs are typically connected at sites on their arms called chiasmata, and sister-chromatid cohesion along the chromosome arms is believed to be important for the maintenance of chiasmata (Maguire, 1974; Maguire, 1993). With the onset of anaphase I, this arm cohesion is lost, but cohesion between the centromeric regions of the sister chromatids is maintained. This cohesion in the centromeric region is required to align the sister chromatids for metaphase II and is released at the beginning of anaphase II. Thus, meiosis is a specialized cell division that requires a two-step release of sister-chromatid cohesion.

The Drosophila protein MEI-S332 has been demonstrated both to be essential for cohesion between sister chromatids and to be localized to chromosomes (Goldstein, 1980; Kerrebrock et al., 1992; Kerrebrock et al., 1995). These cytological studies were performed in spermatocytes. In male meiosis, MEI-S332 localizes to the centromeric regions of meiotic chromosomes and is maintained there through the metaphase I/ anaphase I transition (Kerrebrock et al., 1995). MEI-S332 is observed on chromosomes in metaphase II but is no longer detectable

with the commencement of anaphase II, the time when cohesion between sister chromatids is released. The protein is required primarily for proper segregation during the second meiotic division, since by genetic assays, *mei-S332* mutant males and females have nearly normal segregation during the first meiotic division and high levels of missegregation during the second meiotic division (Davis, 1971; Goldstein, 1980; Kerrebrock et al., 1992). Precociously separated sister chromatids are observed in *mei-S332* spermatocytes in late anaphase I, suggesting that MEI-S332 is vital for centromeric cohesion after the metaphase I/ anaphase I transition (Goldstein, 1980; Kerrebrock et al., 1992). Previous studies have not described the localization of MEI-S332 during female meiosis.

The structure of the meiotic chromatin and the meiotic spindle differs between the sexes in *Drosophila melanogaster* (for review see Orr-Weaver, 1995), so it cannot be assumed that localization of MEI-S332 is the same in both spermatocytes and oocytes. In females, but not in males, synaptonemal complex forms during prophase and reciprocal exchange occurs, resulting in the chiasmata that are assumed to hold homologs together. In males, pairing sites hold the homologs together without synaptonemal complex or reciprocal exchange between the homologs (for review see McKee, 1996). Another significant difference is that the oocyte metaphase I spindle is thought to be organized by the chromatin rather than by centrosomes (Theurkauf and Hawley, 1992), and this function could require that the meiotic chromosomes have a different structure in females. Finally, oocytes arrest during metaphase I, while spermatocytes normally do not, and this requires cohesion to be maintained longer. Differences between meiosis in male and female Drosophila could impact MEI-S332 localization. Moreover, the existence of alleles that affect male and female meiosis with different severity suggests that there must be some differences in MEI-S332 mechanism between the sexes (Kerrebrock et al., 1992). Whereas Drosophila male meiosis has several unusual features, Drosophila female meiosis is more typical of meiosis in most eukaryotes; thus localization of MEI-S332 in oocytes is of particular interest.

Sister chromatids are believed to be held together by proteins until anaphase (for review see Bickel and Orr-Weaver, 1996). The cohesive proteins that hold sister chromatids together could dissociate or could be degraded at the time when the chromatids separate. Studies in both yeast and Xenopus extracts have shown that release of cohesion is dependent on proteolysis of some substrates by the cyclin degradation machinery, the Anaphase Promoting

Complex (APC: Funabiki et al., 1996; Holloway et al., 1993; Irniger et al., 1995). This complex could directly proteolyze the cohesive proteins at the chromosomes, or indirectly promote sister-chromatid separation by degrading inhibitors of anaphase. Recent work in budding yeast demonstrates that the Pds1p protein, which acts as an inhibitor of separation, is degraded by the APC at the initiation of anaphase (Cohen-Fix et al., 1996; Yamamoto et al., 1996; Yamamoto et al., 1996). A second protein more integrally involved in cohesion, the Mcd1p/Scc1p protein, has also been identified (Guacci et al., 1997; Michaelis et al., 1997). Mcd1p localizes to mitotic chromosomes and dissociates at the metaphase/anaphase transition, but its degradation is slow, and the protein persists after anaphase. Thus, both dissociation and degradation may play important roles in the release of sister-chromatid cohesion. Although the cohesion protein MEI-S332 is not observed on the chromatids after the sister chromatids separate during meiosis II, it is not known whether the protein simply dissociates or is degraded.

Here we look at the localization of MEI-S332 during meiosis in females, and we find that, as in males, the protein disappears from centromeres at anaphase II. The fate of MEI-S332 at the metaphase II/ anaphase II transition is examined using Western blots, and we find that MEI-S332 is not degraded detectably at that time. Since the protein is not degraded, we examine its localization during embryonic mitoses. Although centromeric cohesion also occurs in mitosis, *mei-S332* is not essential for mitotic divisions (Kerrebrock et al., 1992; Kerrebrock et al., 1995). Strikingly, we find that the MEI-S332 protein is localized to the centromeric regions of mitotic chromosomes in the embryo.

METHODS

Fly Strains

In the studies of MEI-S332-GFP localization in oocyte meiosis, females of genotype *y w P{+mc* 5.6KK *mei-S332+::GFP=GrM}-13; P{w+mc* 5.6KK *mei-S332+::GFP=GrM}-1*, containing four copies of the fusion transgene *mei-S332+::GFP* (Kerrebrock et al., 1995) and two endogenous copies of *mei-S332+*, were used. (The insertion of the transgene on the X chromosome is named *P{GrM}-13;* the insertion on chromosome 2 is named *P{GrM}-1.*) For localization of MEI-S332-GFP in embryos, mothers of the genotype described above or mothers carrying only two copies of the fusion transgene *mei-S332+::GFP* in the *y; mei-S332^7/Df(2R)X58-6* background were used. The latter flies were generated by crossing *y w P{GrM}-13; cn mei-S332^7 px sp/SM1* females to *y w P{GrM}-13/y+Y; Df(2R)X58-6 pr cn/SM1* males.

In studying the MEI-S332 levels in oocytes before and after activation (Fig. 4-3C), *y w* females were used. Embryos and oocytes from *y; pr cn mei-S332^7 bw sp/Df(2R)X58-6, pr cn* and *y; pr cn mei-S332^7 bw sp/cn mei-S332^7 px sp* females were used as negative controls for the anti-MEI-S332 peptide antibodies (Kerrebrock, et al., 1992 and see below). *Oregon-R* (wild type) was used as the negative control for GFP fluorescence microscopy and positive control for Western blot analysis (Fig. 4-3B). For protein extracts from overexpressing oocytes, oocytes were obtained from females carrying 6 copies of the *mei-S332+* gene (two endogenous copies and 4 copies from homozygous insertions of *P{w+mc* 5.6 KK *mei-S332+}* on the second and third chromosomes; Kerrebrock et al., 1995). In all the *mei-S332* transposons the gene was expressed from the normal genomic regulatory regions.

Meiosis in Activated Eggs

The cytology of activated eggs was performed essentially as described in Page and Orr-Weaver (1997) with changes in the fixation conditions to preserve the GFP fluorescence. 300 females of genotype *y w P{GrM}-13; P{GrM}-1* were fattened on wet yeast for several days. Flies were disrupted in IB (55 mM NaOAc, 40 mM KOAc, 110 mM sucrose, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM HEPES, final pH 7.4) in a blender, and oocytes were isolated by filtration and gravity settling. This isolation step took 10-11 minutes. Oocytes were activated by the addition of AB (3.3 mM NaH_2PO_4, 16.6 mM KH_2PO_4, 10 mM NaCl, 50 mM KCl,

5% PEG 8000, 2 mM $CaCl_2$, final pH 6.4) for a five-minute incubation, and then the buffer was changed to ZAB (9 mM $MgCl_2$, 10 mM $MgSO_4$, 2.9 mM NaH_2PO_4, 0.22 mM NaOAc, 5 mM glucose, 27 mM glutamic acid, 33 mM glycine, 2 mM malic acid, 7 mM $CaCl_2$, final pH 6.8) for an additional incubation of 10 minutes (for anaphase I and metaphase II) or 25 minutes (for anaphase II and the post-meiotic interphase). Eggs with cross-linked vitelline membranes, a hallmark of activation, were selected by a 3-minute incubation in 50% Clorox bleach, and fixed in 8% EM-grade, MeOH-free formaldehyde (Ted Pella Inc.) in cacodylate buffer (100 mM cacodylic acid, 100 mM sucrose, 40 mM KOAc, 10 mM NaOAc, 10 mM EGTA, pH to 7.2 with KOH; Theurkauf, 1994) for 10-15 minutes, and washed in PBST (PBS with 0.3% Triton X-100) containing approximately 1% BSA to prevent sticking to glassware. Vitelline membranes were removed by rolling the fixed eggs between two microscope slides (Theurkauf, 1994), again using PBST/BSA as a lubricant. Eggs were incubated in 1% RNase A (boiled to destroy DNase activity) for 20 minutes, and then incubated with 1 µg/ml propidium iodide (Sigma) for 30 minutes. Samples were mounted in Vectashield containing propidium iodide (Vector Laboratories).

Tubulin Immunofluorescence

Oocytes were prepared using the protocol described by Theurkauf (1994) for isolation and fixation of egg chambers. Tubulin was labeled using two anti-tubulin rat monoclonal antibodies, YL1/2 and YOL1/34 (Sera-Lab), overnight at room temperature at a dilution of 1:5 in 0.1% BSA in PBST, followed by a 3-hour incubation with a Texas Red-conjugated donkey anti-rat antibody (Jackson Immuno Research Laboratories) at room temperature at a dilution of 1:200. The oocytes were further stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) at 1 µg/ml in PBS for 10 minutes, followed by two 15-minute rinses in PBS before mounting in 50% glycerol.

MEI-S332-GFP Localization in Embryos

Embryos were collected for 2.5 hours from females of the genotype *y w P{GrM}-13; P{GrM}-1.* The embryo in Figure 4-5 was from a 4-hour collection from females of the genotype *y w P{GrM}-13; cn mei-S332^7 px sp/Df(2R)X58-6 pr cn. Oregon-R* embryos were used as a control for background autofluorescence.

Embryos were dechorionated in 50% bleach, and fixed for 30 minutes in 8% MeOH-free formaldehyde in cacodylate buffer (see above). After washing in PBS,

embryos were rolled out of their vitelline membranes between 2 glass slides (Theurkauf, 1994). To stain for DNA, two methods were used. Embryos in Figure 4-4A, C, and D were treated with 1 mg/ml RNase A for 30 minutes, stained with 1 μg/ml propidium iodide for 30 minutes, and mounted in Vectashield with propidium iodide (Vector Laboratories). The embryos shown in Figures 4-4B, 4-4E, and 4-5 were stained with DAPI at 1 μg/ml in PBS for 10 minutes, followed by two 15-minute rinses in PBS before mounting in 50% glycerol.

Microscopy

Two kinds of epifluorescence microscopy were used in our investigations. Conventional epifluorescence microscopy was performed using either a Nikon Optiphot-2 microscope or a Nikon Eclipse E800 equipped with a Nikon 60x oil objective. A Photometrics CE200A cooled CCD video camera was used to photograph images. The images were further processed with the CELLscan 2.0 system (Scanalytics) to create volume views from focal planes separated by 0.25 μm. 32 focal planes are shown for the oocyte images in Figure 4-2, 45 focal planes for the rosette in Figure 4-4B, 20 focal planes for the mitotic interphase nucleus in Figure 4-4E, and 7 focal planes for the images in Figure 4-5. Chromatin and MEI-S332-GFP in Figures 4-1A-G, 4-4A, 4-4C, and 4-4D were visualized on a BioRad MRC 600 confocal laser scanning head equipped with a krypton/argon laser, mounted on a Zeiss Axioskop microscope, with 20x, 40x, and 40x oil Plan Neofluar objectives. In some cases, optical sections were taken and projected into a single plane. All images were further processed, colorized, and merged using Adobe Photoshop 3.0 on a Macintosh Power PC.

Western Blot Analysis

The rabbit anti-MEI-S332 antibodies (Covance) were generated against a C-terminal MEI-S332 peptide conjugated to keyhole limpet hemacyanin. This 15-mer peptide (residues 386-400), (C)KNKLRNGSKGKAKAK, was chosen as the antigen because of the availability of the *mei-S332*^{7} allele, which lacks the C-terminal region of the protein because of a nonsense mutation at residue arg^{293} (Kerrebrock et al., 1995) and, hence, provides a negative control for the antibodies. The anti-peptide antibodies were affinity purified from rabbit serum using GST-MEI-S332 fusion protein bound to immobilon-P strips. The antibodies were eluted from the strips by acid elution buffer (5mM glycine-HCl pH2.5, 150mM NaCl) and immediately neutralized by 1M $NaPO_4$ buffer pH8. The GST-MEI-S332

fusion protein was generated by cloning a 1.35 kb *Bam*HI-*Eco*RI *mei-S332* cDNA fragment in frame with GST in the pGEX-4T-3 expression vector (Pharmacia). The resulting pGEX.MEI plasmid allowed for expression of the full-length MEI-S332 protein, fused to GST at the N-terminus, in BL21(λDE3)pLysS cells.

Embryonic extracts were made by dechorionating *Oregon-R* embryos in 50% Clorox bleach and homogenizing in 1 x urea sample buffer (USB: 8 M urea, 2% SDS, 5% β-mercaptoethanol, 100 mM Tris pH7.6, and 5% Ficoll) at 5:1 USB:embryo (v/v). Oocyte extracts were made from mature oocytes isolated as described in Page and Orr-Weaver (1997). Females were fattened for 3-5 days with yeast before blender isolation. Oocytes were homogenized in 1 x urea sample buffer at 3:1 USB:oocyte (v/v). Ovary extracts were made by dissecting previtellogenic, immature ovaries or mature ovaries in PBS from newly eclosed females or females that were fattened on yeast for 3 days, respectively, and homogenizing pooled ovaries in 1 x USB (approximately 1 μl buffer/ovary). All protein extracts were cleared by centrifugation, frozen in liquid nitrogen, and stored at -80°C.

For the analysis of MEI-S332 levels in oocytes before and after activation, oocytes were isolated in IB, in either the presence or absence of 100 μg/ml cycloheximide (Fluka), from 300 *y w* females fattened on wet yeast for 3 days, as described above. After isolation, half of the oocytes were fixed by immersion in MeOH (unactivated) and the other half were activated in AB and ZAB in either the presence or absence of 100 μg/ml cycloheximide, as described above. The total incubation time in AB+ZAB was 60 minutes. These activated eggs were then fixed by incubation in MeOH. After several hours fixation in MeOH at room temperature, oocytes and eggs were rehydrated in PBS. Rehydrated samples were mixed with 1:1 EB:4 x Laemmli Sample Buffer (EB: 10 mM Tris 7.5, 80 mM Naβ-glycerophosphate pH 7.5, 20 mM EGTA, 15 mM $MgCl_2$, 2 mM Na_3VO_4, 1 mM benzamidine, 1 mM sodium metabisulfite, 0.2 mM PMSF) by crushing with the melted tip of a glass pipette. The ratio of sample to buffer added was 1:4 (v/v). Samples were boiled for 15 minutes, cleared by centrifugation, and frozen in a dry ice/MeOH bath. Control extracts for this experiment were made by isolating and fixing unactivated oocytes from *pr cn mei-S332^7 bw sp/cn mei-S332^7 px sp* and *Oregon-R* females as above. A cross-reacting band on the Western blot, just below the MEI-S332 signal, is also present in the *mei-S332^7* negative control, and is perhaps an artifact of this sample preparation.

Protein extracts were separated on 12% 150:1 (acrylamide:bis-acrylamide) gels and blotted onto immobilon-P membranes (Millipore). About 200 µg of total protein was loaded per lane, and Ponceau S staining was used to verify equivalent protein loading prior to immunoblotting. Blots were blocked in 5% nonfat dry milk and 2% BSA in TBST (0.01 M Tris pH7.5, 0.9% NaCl, and 0.1% Tween 20) for one hour at room temperature and then incubated overnight at room temperature with affinity-purified anti-MEI-S332 peptide antibodies diluted at 1:40 in the block solution. Alkaline phosphatase-conjugated anti-rabbit secondary antibodies (Promega), diluted 1:7500 in the block solution, were used to detect bound anti-peptide antibodies. The MEI-S332 protein was visualized using the BCIP/NBT color development substrate (Promega). Although it is predicted to be 44 kDa, the MEI-S332 protein migrates as a 55 Mr band.

RESULTS

MEI-S332 localizes to centromeric regions in oocytes

Although the localization of MEI-S332 has been determined in spermatocyte meiosis (Kerrebrock et al., 1995), the differences between male and female meiosis in *Drosophila melanogaster* and the existence of *mei-S332* alleles that affect the two sexes with different severity led us to ask where MEI-S332 is localized in oocyte meiotic divisions. Specifically, we asked whether it localizes to meiotic centromeres, and if so, what is the fate of the protein when the sisters separate at anaphase II.

To visualize the MEI-S332 protein in oocytes, we used a fusion of GFP to the N-terminal end of *mei-S332* (*mei-S332*$^{+}$*::GFP*) that has been shown to complement fully the mutant phenotype in both males and females (Kerrebrock et al., 1995). In Drosophila, mature oocytes arrest at metaphase I with a tapered spindle and an elongated nucleus. We examined fixed oocytes stained for DNA and observed that MEI-S332-GFP was present in two caps at opposite ends of the oocyte nucleus (Fig. 4-1A). The orientation of the caps with respect to the morphology of the oocyte nucleus suggested that these caps were facing the poles of the metaphase I spindle, and tubulin staining later confirmed this interpretation (see below). Since it has been shown that the centromeric regions of chromosomes are positioned on opposite sides of the chromatin mass during the metaphase I arrest in Drosophila oocytes (Dernburg et al., 1996), it was likely that caps of MEI-S332-GFP represented centromeric localization.

We wanted to determine what happens to these caps of MEI-S332 when the meiotic cell cycle resumes after the oocyte arrest. In particular, we sought to observe the localization of the protein during anaphase I, when centromeric localization would be most apparent, and observe what happens to the protein at the metaphase II/ anaphase II transition when the sister chromatids separate. Historically, it has been difficult to observe any of the stages of female meiosis that follow the metaphase I arrest in Drosophila oocytes, but recent advances in egg activation in vitro now allow all the stages of meiosis to be examined (Page and Orr-Weaver, 1997). Accordingly, oocytes from mothers carrying the *mei-S332*$^{+}$*::GFP* transgene were activated in vitro to complete meiosis, then fixed and stained for DNA. Oocytes in anaphase I had 8 pairs of sister chromatids, four on each side, as is expected since the haploid chromosome number in Drosophila is four. Such oocytes also had 8 dots of MEI-S332-GFP visible at the leading edges of

Figure 4-1. MEI-S332-GFP localizes to centromeric regions of female meiotic chromosomes until anaphase II.

MEI-S332-GFP is shown in green and chromatin in red. Oocytes were isolated from females carrying four copies of the *mei-S332+::GFP* transgene, activated in vitro, fixed, and stained with propidium iodide. Images were collected using confocal microscopy. Bar is approximately 5 µm.

(A) Unactivated stage-14 oocytes arrested in metaphase I localize MEI-S332-GFP at two discrete sites on the opposite ends of the condensed chromosomes.

(B) At the onset of anaphase I, 8 dots of MEI-S332-GFP are visible at the leading edges of the separating anaphase chromosomes, one per pair of sister chromatids, with the fourth chromosomes closest to the poles. Chromosome *4* in Drosophila is very small and sometimes difficult to visualize.

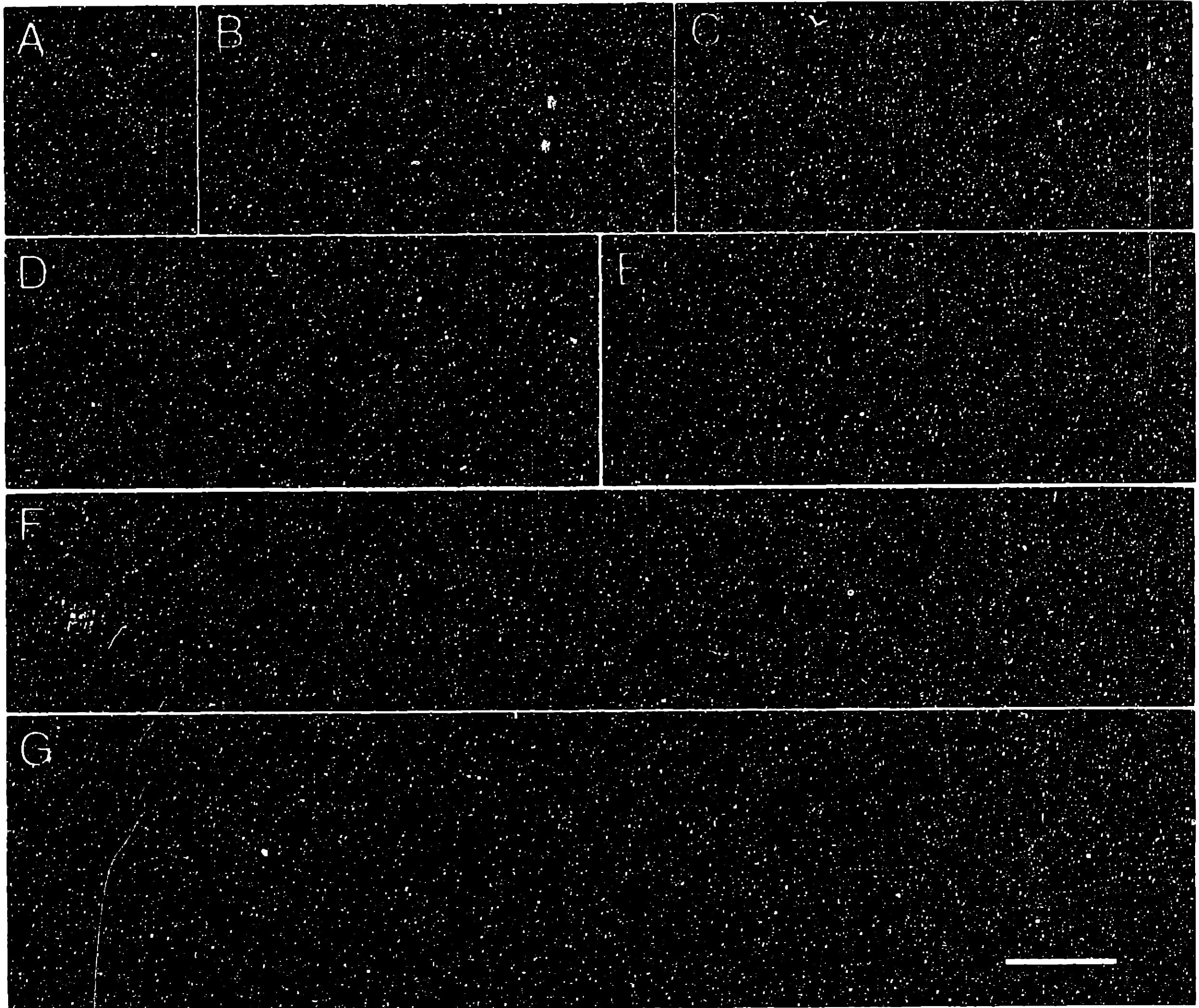
(C) In late anaphase I, MEI-S332-GFP is still detected at the leading edges the chromosomes, which become shorter and rounder as they approach the poles.

(D) Between the first and second meiotic divisions, two clusters of 3-4 chromatin balls are observed. Each ball most likely represents a pair of sister chromatids and is associated with a dot of MEI-S332-GFP.

(E) In metaphase II, the chromatin balls move together to form metaphase plates, and MEI-S322-GFP localizes to the middle of the chromatin.

(F) When sister-chromatid cohesion is released at anaphase II, the sister chromatids separate. MEI-S332-GFP is no longer detectable on meiotic chromosomes.

(G) During the post-meiotic interphase, MEI-S332-GFP is not visible on the decondensed chromosomes.


A
B
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G

the separating chromosomes, one per pair of sister chromatids (Fig. 4-1B). The observation that each pair of sister chromatids had MEI-S332 at their leading edge argued strongly that MEI-S332 is localized at the centromeric regions of chromosomes in female meiosis. MEI-S332-GFP was continually visible on the chromosomes between anaphase I and metaphase II (Fig. 4-1B-E and see below). When sister-chromatid cohesion was released at anaphase II, the sister chromatids separated, and for the first time during the meiotic divisions, MEI-S332 was not observed on the chromosomes (Fig. 4-1F). After the meiotic divisions, the chromatin decondensed into four nuclei (three polar bodies and one pronucleus) in the post-meiotic interphase. MEI-S332-GFP was not detectably localized during the post-meiotic interphase (Fig. 4-1G).

The cytology of nuclei between the meiotic divisions has been difficult to observe in oocytes. Indeed, even with the in vitro activation system, the lack of familiar cytological landmarks between anaphase I and metaphase II has meant that it was still unknown what happened to chromosome morphology between the divisions. Although it is known that in Drosophila male meiosis the telophase I nuclei decondense and then recondense for meiosis II (Cenci et al., 1994), it was unclear whether such decondensation occurred in Drosophila females. In experiments activating hundreds of *mei-S332*$^{+}$*::GFP* transgenic oocytes, we never observed oocytes with only two decondensed nuclei, in agreement with our unpublished observations with oocytes from non-transgenic flies. Thus it appears that Drosophila oocyte nuclei remain condensed throughout meiosis until telophase II.

Since it was clear from the early anaphase I figures that MEI-S332-GFP labels the centromeric regions of oocyte meiotic chromosomes (Fig. 4-1B), we were able to use it as a tool in deducing the order of events in chromatin remodeling between anaphase I and metaphase II. We observed that late anaphase I chromosomes appear to become shorter and rounder as they approach the poles, but despite these morphological changes they could always be identified by the leading edge of MEI-S332-GFP at the centromere (Fig. 4-1C). Between the divisions, the chromosomes rounded up and formed two clusters of three or four individual balls of chromatin (Fig. 4-1D). Each ball was associated with a dot of MEI-S332-GFP, but the dots were no longer oriented at the leading (outside) edge of the chromosomes. We think it likely that each ball represents the sister chromatids of each of the three large chromosomes, with the small fourth chromosome only sometimes visible. Metaphase II was evident when the

clusters of chromatin balls compacted to form metaphase plates, usually parallel to each other, with MEI-S332-GFP in the middle of the compacted chromatin (Fig. 4-1E). Often, as in Figure 4-1E, the two nuclei were slightly out of synchrony. Even though there is no decondensation between the meiotic divisions, a series of interesting changes occurs in chromosome morphology between anaphase I and metaphase II.

When does MEI-S332 localize to centromeres?

In spermatocytes, MEI-S332 protein is observed in the cytoplasm during prophase I, and it is localized to the chromosomes as they compact for prometaphase I (Kerrebrock et al., 1995). We examined when and how MEI-S332 is localized prior to metaphase I in oocytes, since there are marked differences between spermatocytes and oocytes during prophase I. The origin of the cytoplasm in oocytes differs from that in spermatocytes, since much of it is created in the nurse cells, and the volume of cytoplasm is much greater in oocytes than in spermatocytes. Another important difference is that synaptonemal complex is seen on oocyte chromosomes but not on spermatocyte chromosomes. Sex-specific differences in the origin and amount of cytoplasm or in the structure of the meiotic chromosomes suggested that the timing of MEI-S332 localization should be examined in oocytes to see if it differed from spermatocytes.

To examine MEI-S332 localization in oocytes during early developmental stages, ovaries were dissected from females carrying the *mei-S332⁺::GFP* transgene, fixed and stained for DNA (data not shown). MEI-S332-GFP was not observed in egg chambers during prophase I, corresponding to oocyte development through stage 12, either in the cytoplasm or on the condensed meiotic chromosomes in the karyosome. Multiple foci of MEI-S332-GFP were first observed on the meiotic chromatin after the chromatin compacted into the small round mass characteristic of prometaphase I. Using egg chamber morphology to judge developmental stage, we determined that these foci first appeared in stage 13. By stage 14, MEI-S332-GFP was observed in two caps on either side of the nucleus (Fig. 4-1A and see below).

Since the meiotic spindle is organized shortly after the chromatin compacts, we further characterized the localization of MEI-S332-GFP with respect to formation of the spindle by isolating stage 13 and 14 oocytes and labeling both the DNA and tubulin. After compaction of the chromatin in stage 12, the nuclear envelope breaks down and short microtubule fibers captured by the chromatin

subsequently coalesce into a bipolar spindle during stage 13 (Theurkauf and Hawley, 1992). The earliest stage at which MEI-S332-GFP was observable was coincident with the beginning of spindle formation. A small number of dots of MEI-S332-GFP were distributed throughout the chromosomal mass (Fig. 4-2A). When spindles appeared more bipolar and elongated, typical of late stage 13 and stage 14 oocytes, the MEI-S332-GFP foci were more clearly combined into caps on the ends of the chromatin mass that face the spindle poles (Fig. 4-2B,C).

The metaphase II/anaphase II transition

In both female and male meioses MEI-S332 was not visible on the sister chromatids after they separated at anaphase II; consequently we investigated what happened to the protein when sister-chromatid cohesion was released. In yeast and Xenopus mitosis, an inhibitor of sister-chromatid separation is degraded by the cyclin destruction machinery at the metaphase/anaphase transition (Cohen-Fix et al., 1996; Holloway et al., 1993; Irniger et al., 1995). Since MEI-S332 is essential for sister-chromatid cohesion, it seemed plausible that it might be degraded at the metaphase II/ anaphase II transition.

To study protein levels directly, we generated polyclonal rabbit antibodies against a peptide corresponding to the C-terminal fragment of the MEI-S332 protein (Fig. 4-3A). Affinity-purified antibodies recognized a band of approximately 55 Mr on a Western blot of ovary and oocyte extracts (Fig. 4-3B). This band was absent in extracts made from *mei-S332*^{7} oocytes and ovaries (Fig. 4-3B, lanes 2 and 3). Extracts from *mei-S332*^{7} homozygotes and hemizygotes provided a critical negative control, as this mutation creates a nonsense codon that prematurely truncates the protein so that it lacks the epitope for the C-terminal peptide antibodies (Fig 4-3A). As additional evidence that the identified band is MEI-S332, we probed extracts from transgenic ovaries that had 4 extra copies of a genomic *mei-S332*$^+$ fragment in addition to the two endogenous copies, and we found that the band was significantly more intense (Fig. 4-3B, lane 1). These data lead us to conclude that the peptide antibodies recognize the MEI-S332 protein as a 55 Mr band on Western blots. This protein migrates during electrophoresis as a 55 Mr band even though its predicted size is 44 kDa.

To determine whether MEI-S332 is degraded at the metaphase II/ anaphase II transition we analyzed in vitro activated oocytes. Sixty minutes after activation, eggs can be selected so that 95-99% have completed meiosis (Page and Orr-Weaver, 1997). We compared MEI-S332 protein levels between extracts of

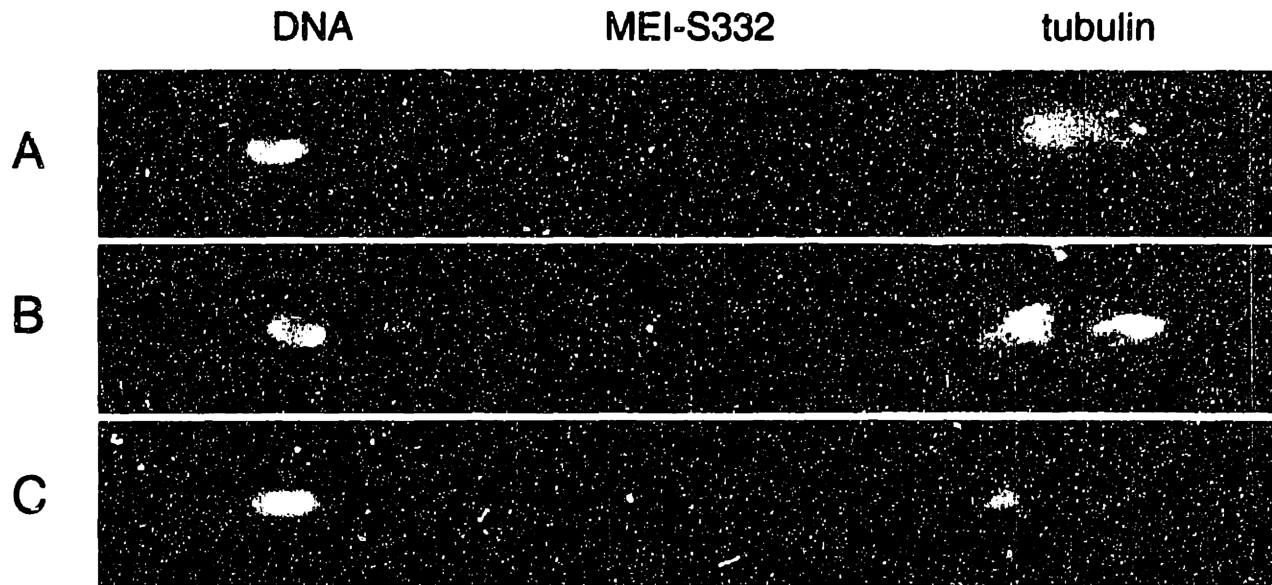


Figure 4-2. MEI-S332-GFP assembly onto female meiotic chromosomes correlates with spindle formation.

DNA staining is shown on the left, MEI-S332-GFP in the middle, and anti-tubulin staining on the right. Oocytes were isolated from females carrying four copies of the *mei-S332⁺::GFP* transgene, fixed, and stained with anti-tubulin antibodies and DAPI. Images were collected using a CCD camera. Bar is approximately 5 µm.

(A) MEI-S332-GFP is first observed on the meiotic chromosomes at multiple discrete sites before the formation of a bipolar spindle.

(B) As the spindle becomes increasingly elongated and bipolar, the discrete dots of MEI-S332-GFP begin to cluster at opposite ends of the chromatin mass.

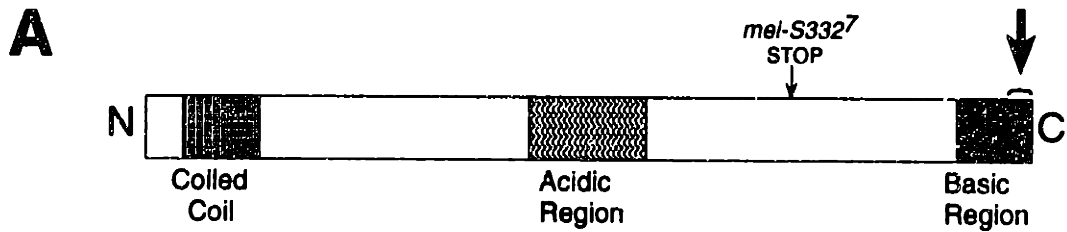
(C) When the spindle is fully elongated, MEI-S332-GFP is observed in two caps at the opposite ends of the chromatin mass, aligned with the bipolar spindle.

Figure 4-3. The MEI-S332 protein is present in embryos and is not globally degraded at the metaphase II/ anaphase II transition.

(A) A schematic of the MEI-S332 protein. Anti-MEI-S332 antibodies were generated against a C-terminal 15-amino acid peptide of MEI-S332 (large arrow). Tissues from *mei-S332*^{7} flies were used as negative controls for the antibodies because the *mei-S332*^{7} mutation generates a truncated form of the protein that lacks the epitope for the C-terminal peptide antibodies.

(B) The MEI-S332 protein, predicted to be 44kDa, is recognized as a 55 Mr band on Western blots by affinity-purified anti-MEI-S332 peptide antibodies. Higher levels of MEI-S332 (lane 1) are seen in oocytes isolated from females carrying 6 copies of the *mei-S332* $^+$gene (two endogenous copies and 4 copies of a genomic fragment). MEI-S332 is present in previtellogenic ovaries (lane 4), mature ovaries (lane 5), 0-2 hour embryos (lane 6), and 2-4 hour embryos (lane 7). There appear to be different mobility forms of MEI-S332 in embryos. As expected, the 55 Mr band is not detected in *mei-S332*^{7} oocytes and ovaries (lanes 2 and 3).

(C) MEI-S332 protein levels remain essentially unchanged in activated eggs that have completed meiosis (compare lanes 3 and 4). Although MEI-S332 is no longer detectable on the chromosomes when sister-chromatid cohesion is lost, it is not degraded globally. Protein levels remain unchanged when meiosis is completed in the presence of the translational inhibitor cycloheximide (compare lanes 5 and 6). *Oregon-R* and *mei-S332*^{7} unactivated oocytes were used as positive and negative controls, respectively, for the antibodies (lanes 1 and 2). The lower non-specific band, probably an artifact of this sample preparation, is not MEI-S332 as it is still present in extracts from *mei-S332*^{7} oocytes (lane 2).


A
mei-S332^7
STOP
N
C
Coiled
Coil
Acidic
Region
Basic
Region

B
mei-S332 7
wild-type
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ovaries
immature ovaries
ovaries
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2-4 hour embryos
66.2 kD
1
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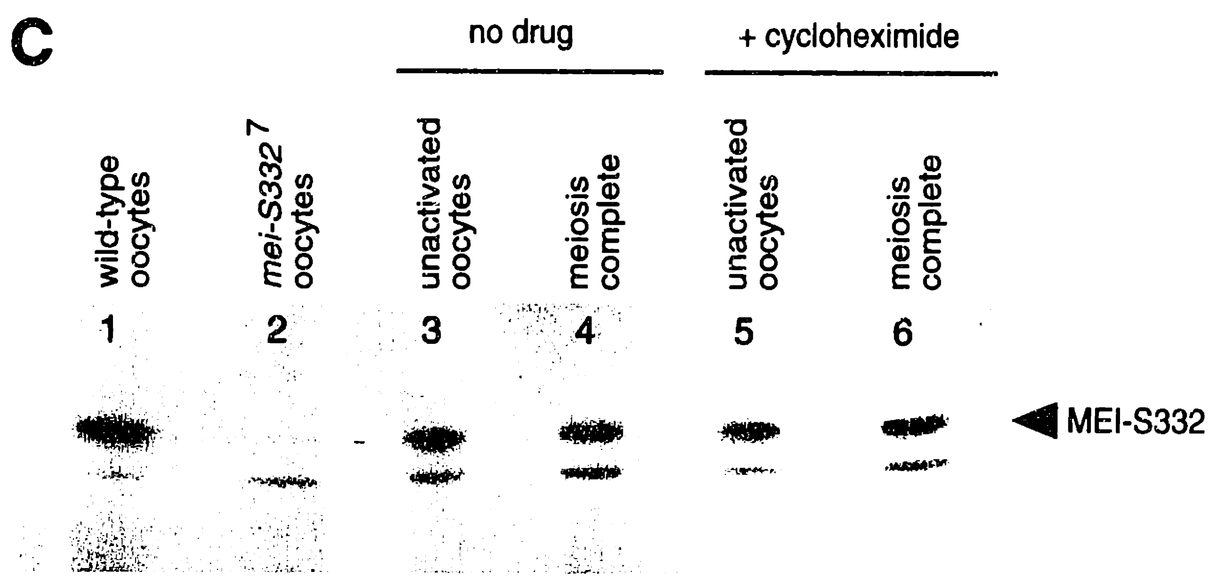
7
MEI-S332
45.0 kD


C
no drug
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wild-type oocytes
mei-S332 7 oocytes
unactivated oocytes
meiosis complete
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1
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MEI-S332

unactivated oocytes, which have MEI-S332 localized to the chromosomes (Fig. 4-1A), and extracts of eggs activated for 60 minutes, which have passed through the metaphase II/ anaphase II transition. On Western blots, these protein levels remained essentially unchanged (Fig. 4-3C, lanes 3 and 4), a result that was repeated several times. This suggests that although the protein dissociated from the chromosomes at anaphase II, it was not degraded.

Although the total levels of MEI-S332 remained constant before and after meiosis was completed, we were concerned that continuing translation of new MEI-S332 protein might mask protein degradation. To address this concern, we activated oocytes in the presence of the translational inhibitor cycloheximide. Metabolic labeling experiments have demonstrated that oocytes activated in the presence of cycloheximide have protein synthesis inhibited to about 5% of wild-type levels, but that about 95% of oocytes still complete meiosis under these conditions, arresting at the post-meiotic interphase (Page and Orr-Weaver, 1997). Western blotting of extracts from arrested, unactivated oocytes incubated in cycloheximide compared to extracts from oocytes activated in the presence of cycloheximide further demonstrated that there was no detectable degradation of MEI-S332 during meiosis, suggesting that it instead delocalized (Fig. 4-3C, lanes 3-6).

MEI-S332 during mitosis

The phenotype of *mei-S332* mutants was previously shown to be exclusively meiotic and not mitotic: no cytological defect has been detected in proliferating tissues, mutants are completely viable, and no increase in somatic clones from mitotic errors is observed (Kerrebrock et al., 1992; Kerrebrock et al., 1995). However, our finding that the protein was not degraded at anaphase II led us to ask whether the protein persisted in the developing embryo. We examined extracts from wild-type oocytes and embryos by Western blotting, and we found significant amounts of MEI-S332 in a collection of embryos of ages 0-2h (Fig. 4-3B, lane 6). The protein level appeared to increase in populations of embryos of ages 2-4h (Fig. 4-3B, lane 7), suggesting that MEI-S332 did not merely persist into embryogenesis, but could be playing a role there. Additionally, we noted that there appeared to be different mobility forms of MEI-S332, an observation that is currently under investigation.

We used the *mei-S332*$^{+}$*::GFP* transgene to determine whether MEI-S332 could localize onto chromosomes in the embryo, and we observed persistent

localization of the protein on polar body rosettes (Fig. 4-4A,B). Chromosomes from the unused meiotic products are pulled into a radial formation by a sphere of tubulin, after replicating and condensing into a metaphase-like state. These are found in the anterior dorsal quadrant of early embryos, typically fused so that there exist only one or two rosettes (Foe et al., 1993). MEI-S332-GFP localized to the condensed chromosomes facing the inside of the rosette, where centromeres are expected to be located (Foe et al., 1993). Moreover, when all the unused meiotic chromosomes have fused into a single rosette formation, the number of chromosomes should be 12, or after replication 24, and we count approximately 24 foci of MEI-S332-GFP in a typical single rosette formation (Fig. 4-4B). As in meiosis, MEI-S332 localized to the apparent centromeric regions of replicated sister chromatids.

MEI-S332-GFP also localized to condensed chromosomes in the early mitotic divisions. Drosophila embryos have 13 syncytial nuclear division cycles before gastrulation. On condensed prometaphase and metaphase chromosomes of these early cycles we observed MEI-S332-GFP in punctate dots resembling those on meiotic chromosomes, consistent with centromeric localization (Fig. 4-4C). These punctate dots were not observed in interphase nuclei (Fig. 4-4D,E). In addition to chromosome localization, diffuse clouds of fluorescence were observed in the vicinity of each mitotic nucleus (Fig. 4-4C). Similar diffuse clouds of MEI-S332-GFP fluorescence were evident near interphase nuclei (Fig. 4-4D,E) and produced a signal brighter than the background autofluorescence in embryos lacking the transgene (data not shown). These clouds of fluorescence may correspond to energids, regions of yolk-free cytoplasm that have been observed in the early cycles of Drosophila embryos (Foe et al., 1993). Immunofluorescence with anti-peptide antibodies confirmed the localization to polar body chromosomes and condensed mitotic chromosomes (data not shown).

In later syncytial divisions, the nuclei migrate to the surface of the embryo and mitosis proceeds in a wave across the embryo. We examined mitotic chromosomes in these easily visualized nuclei to analyze localization of MEI-S332 during the metaphase/anaphase transition in mitosis. To simulate the same *mei-S332* gene dosage as that of wild-type oocytes, embryos from mothers hemizygous for *mei-S332*^{7} and carrying two copies of the *mei-S332*$^{+}$*::GFP* transgene were examined after fixation in formaldehyde and DNA staining. MEI-S332-GFP was observed in bright dots aligned on the metaphase plates with the chromatin (Fig. 4-5A; see arrow for one example). Sometimes much dimmer

Figure 4-4. MEI-S332-GFP localizes to condensed chromosomes in embryos.

MEI-S332-GFP is shown in green and DNA in red. Embryos were collected from females carrying four copies of the *mei-S332+::GFP* transgene, fixed, and stained with either propidium iodide or DAPI. Images in (A), (C), and (D) were collected using confocal microscopy, and images in (B) and (E) were collected using a CCD camera. Bars in A, B, C, and E are approximately 5 μm. Bar in D is approximately 30 μm.

(A) MEI-S332-GFP is present on the polar body rosettes.

(B) A close-up image of a polar body rosette shows punctate MEI-S332-GFP localization on the inside ring of the rosette where centromeres are believed to be pulled to the center. 22 dots of MEI-S332-GFP can be counted in the single rosette found in this embryo.

(C) MEI-S332-GFP localizes to discrete dots on a mitotic metaphase plate, resembling those on meiotic metaphase II chromosomes. In addition, a cloud of diffuse MEI-S332-GFP is observed around each mitotic nucleus.

(D) MEI-S332-GFP is detected in clouds surrounding the interphase nuclei. The nuclei are not centered within the clouds.

(E) A close-up image of the interphase nucleus demonstrates the absence of MEI-S332-GFP localization on the decondensed interphase chromatin.

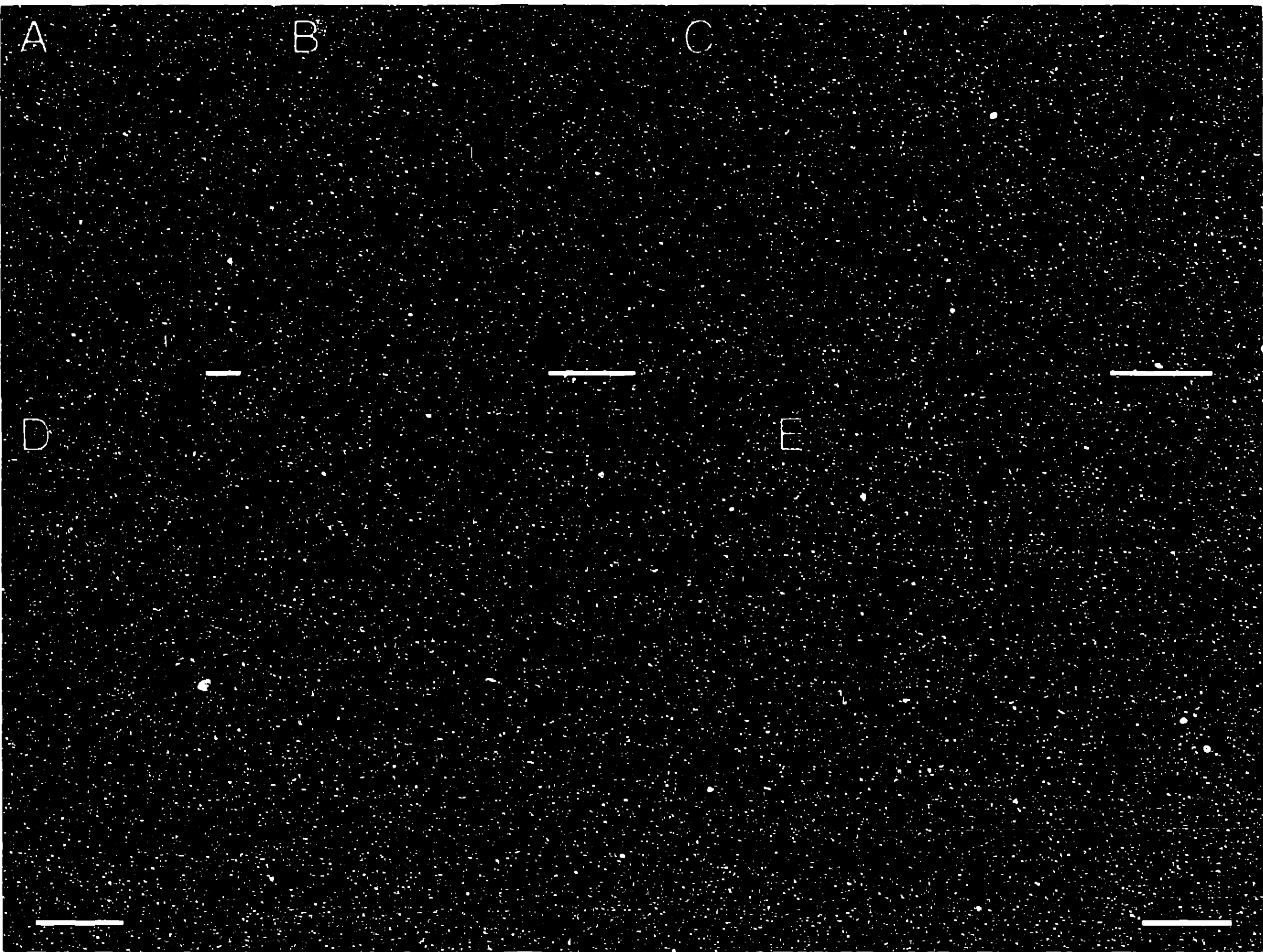
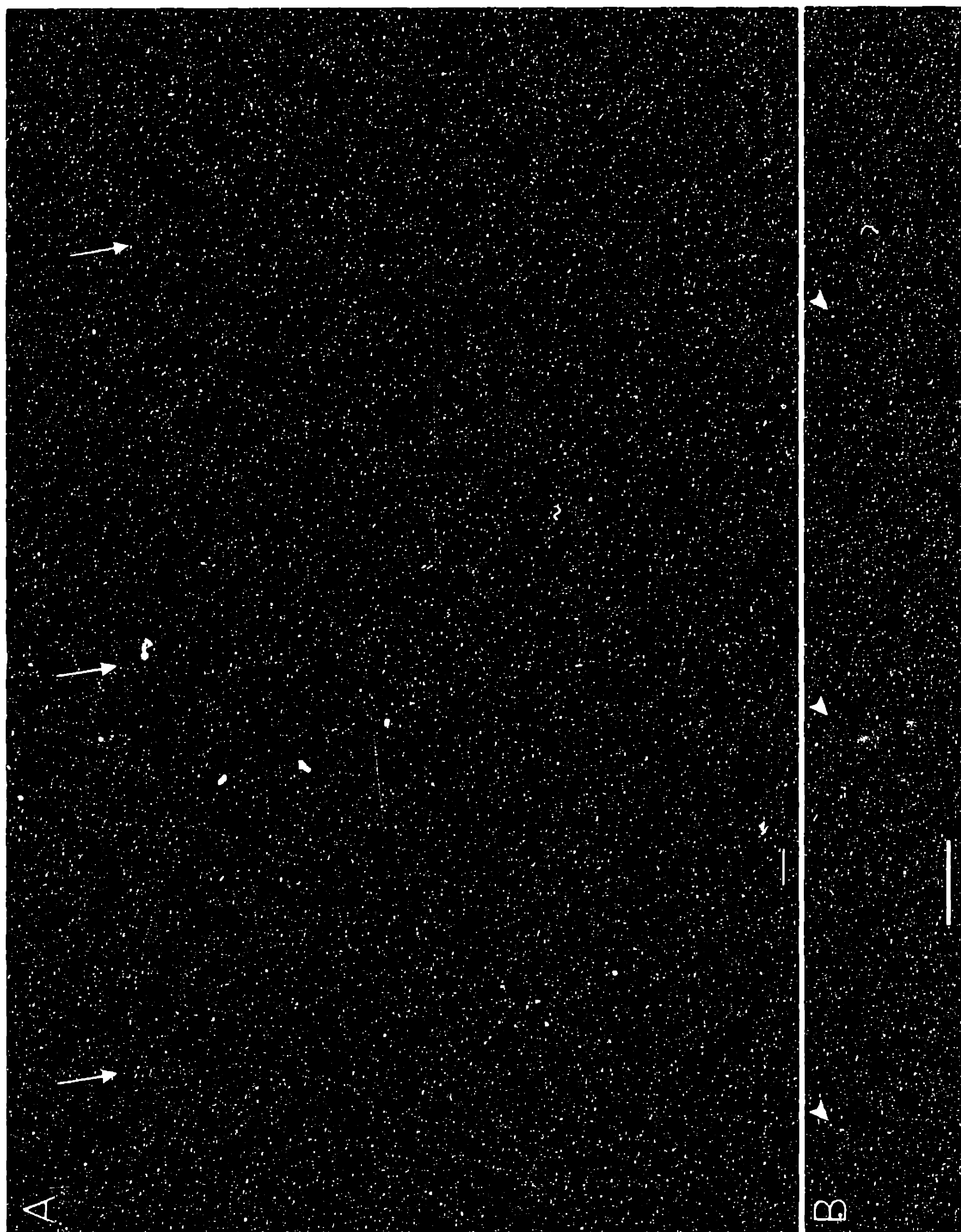

A
B
C
D
E

Figure 4-5. MEI-S332-GFP disappears from centromeres at the metaphase/ anaphase transition in embryos.

MEI-S332-GFP is shown in green, DNA in red. Images are also separated to show individual channels as labeled. Embryos were collected from *mei-S332*^{7} females carrying two copies of the *mei-S332*$^{+}$*::GFP* transgene. Images were collected using a CCD camera. Bars are approximately 5 µm.

(A) A field of syncytial nuclei in a cycle 12 embryo are in the process of mitosis. In each panel, metaphase figures are on the top, anaphase figures in the middle, and late anaphase figures on the bottom. MEI-S332-GFP localizes to discrete dots on the mitotic metaphase plates (arrow shows one example). MEI-S332-GFP is no longer detectable on mitotic chromosomes in late anaphase.

(B) MEI-S332-GFP can be seen at the leading edge of the chromosomes in early anaphase (arrowhead), but it is no longer detectable on mid-anaphase chromosomes.



dots of MEI-S332-GFP were observed on chromosomes in early anaphase (Fig. 4-5B, arrowhead). The residual MEI-S332-GFP was found on the leading edge of chromosomes. By late anaphase and telophase, no MEI-S332-GFP was observed on any of the chromatin. Thus, the metaphase/anaphase transition begins a process of delocalization of MEI-S332. The alignment of the dots on the metaphase plate and the association of residual MEI-S332-GFP with the leading edges of chromosomes strongly suggests that MEI-S332 is localized to the centromeric regions of mitotic chromosomes. Thus, in mitosis as in meiosis, MEI-S332 is localized to the centromeric regions of chromosomes condensed for metaphase, and MEI-S332 begins to dissociate from the chromatin when cohesion is lost and the sister chromatids segregate.

DISCUSSION

In this study we examined the expression and localization of MEI-S332 in Drosophila oocytes and embryos. We found that in oocytes, MEI-S332 localizes to the centromeric region of condensed meiotic chromosomes from prometaphase I until the metaphase II/ anaphase II transition, when sister chromatids separate. This is essentially the same localization pattern as has been observed in spermatocyte meiosis (Kerrebrock et al., 1992). It is striking that although no mitotic phenotype has been observed in *mei-S332* mutants (Kerrebrock et al., 1995), MEI-S332 protein has a similar localization pattern in the early mitotic divisions in the embryo, where it appears bound to condensed chromosomes until the sister chromatids separate at anaphase. On the chromosomes of polar bodies, which are constitutively condensed in a configuration analogous to metaphase, MEI-S332 is consistently observed at the expected centromeric regions. Thus MEI-S332 appears localized to centromeres of metaphase chromosomes in each of these three different cell cycles, and it is dispersed each time sister chromatids separate.

MEI-S332 and the metaphase/anaphase transition

Precisely what happens to MEI-S332 when sister chromatids separate at anaphase is a question of great interest. One possibility is that the protein is degraded at the metaphase/anaphase transition. To test this idea, we examined the levels of MEI-S332 in oocytes before and after the completion of meiosis. We found that even in the presence of cycloheximide to prevent new protein synthesis, the levels of MEI-S332 appeared unchanged before and after the metaphase II/ anaphase II transition. This result demonstrates that on a global level MEI-S332 is not degraded at anaphase II. Although we have not directly examined the question of degradation in mitosis, the observation that MEI-S332 protein is visible in clouds around interphase nuclei strongly supports the idea that it is not degraded on a global level in the developing embryo during the syncytial divisions. Still, we cannot exclude the possibility that centromere-localized protein is locally degraded at either the metaphase II/ anaphase II transition in oocytes or at the mitotic metaphase/anaphase transition. If a subpopulation of MEI-S332 was degraded at anaphase II, however, the amount degraded would have to be insignificant compared to the persisting fraction, since we do not observe any decrease in protein levels by Western blotting.

A second possibility is that dissociation of MEI-S332 from the centromeric regions triggers sister-chromatid separation. An analogous mechanism may occur in the yeast *S. cerevisiae*, because the Mcd1p/Scc1p cohesion protein localized on the chromosomes is not degraded until after anaphase. Instead it is removed from the chromosomes beginning at anaphase (Michaelis et al., 1997; Guacci et al., 1997). Noting that MEI-S332 appears to run as a doublet on Western blots, we speculate that dissociation of MEI-S332 may be regulated at some level by phosphorylation. Consistent with this speculation, the MEI-S332 protein has 30 possible phosphorylation sites recognized by protein kinase C, casein kinase II, cAMP-dependent protein kinase, and tyrosine protein kinase.

There is a third possibility, however, that MEI-S332 may first be inactivated to permit anaphase movement and subsequently dissociate from the chromosomes. This model is supported by our detection of MEI-S332 on the centromeres of chromosomes in early anaphase, although the levels are reduced compared to metaphase. Similarly, some Mcd1p/Scc1p remains localized to the chromosomes in anaphase (Michaelis et al., 1997; Guacci et al., 1997). We cannot distinguish between these two latter models at this point, because it is possible that sufficient amounts of MEI-S332 or Mcd1p/Scc1p dissociate at the metaphase/anaphase transition to permit sister-chromatid separation. Residual levels may then be removed subsequently.

Establishment versus maintenance of sister-chromatid cohesion

In spermatocytes, oocytes, and early embryos MEI-S332 is not detectable on the chromosomes until prometaphase. It is possible that sister-chromatid cohesion is not fully established until this point and that the localization of MEI-S332 marks the establishment of cohesion. It may be the case, however, that cohesion is established immediately after DNA replication. In FISH studies done in yeast, separate signals from the two sister chromatids were not observed until anaphase, indicating that sister chromatids are tightly associated from the time of their replication (Guacci et al., 1994; Guacci et al., 1993). This suggests that cohesion is established during S phase. If this is true, then MEI-S332 may be required to maintain or augment cohesion when spindle forces come into play, rather than to establish cohesion. For example, it may serve to protect and preserve proteins directly attaching the sister chromatids until anaphase.

A mitotic role for MEI-S332?

We were surprised to find that MEI-S332 localizes to mitotic chromosomes in much the same way it localizes to meiotic chromosomes in spermatocytes and oocytes because no function has been ascribed to MEI-S332 in mitosis. The presence of MEI-S332 on mitotic chromosomes is not unique to the early embryonic cycles. MEI-S332 protein is present in dividing larval tissues and can localize to the chromosomes during mitosis (H. LeBlanc, T.T., and T.O-W, unpublished results). We and our colleagues have undertaken careful phenotypic analyses of *mei-S332* mutants in order to determine whether the protein is required for mitosis. Viability studies have demonstrated that *mei-S332* homozygotes and their heterozygous siblings survive equally (Kerrebrock et al., 1992), even when the maternal *mei-S332* contribution is eliminated (H. LeBlanc and T. O.-W., unpublished data). Examinations of large numbers of larval brains, a mitotically active tissue that when squashed flat gives excellent mitotic cytology, demonstrated no significant difference in mitotic index or premature sister-chromatid separation between *mei-S332* hemizygous (*mei-S332/Df*) and wild-type larval brains (Kerrebrock et al., 1995). Furthermore, experiments testing the frequency of chromosome missegregation in the developing wing demonstrated no significant difference between *mei-S332* hemizygotes and their heterozygous siblings (Kerrebrock et al., 1995).

If MEI-S332 is localized to mitotic centromeres, why do we not see a phenotype in *mei-S332* mutants? One possibility is that in mitosis there is redundancy in the mechanisms that hold sister chromatids together. The simplest model for redundancy is that both MEI-S332 and another protein act independently to bind sister chromatids together at the centromeric regions in mitosis, and therefore no phenotype is observed when *mei-S332* is mutated. Currently there are no candidates for such a protein. Although mutations have been characterized in three genes that encode Drosophila centromere-binding proteins, none appear to promote sister-chromatid cohesion. The HP1 and PROD proteins affect centromere condensation and presumably kinetochore function (Kellum and Alberts, 1995; Torok et al., 1997), whereas ZW10 may monitor spindle attachment to the kinetochore (Williams et al., 1996). Another version of this redundancy model is that while MEI-S332 acts at mitotic centromeres to attach sister chromatids, other proteins act along the lengths of the chromatid arms to ensure cohesion and proper orientation with respect to the mitotic spindle. The loss of MEI-S332 would result in the loss of centromeric cohesion, but this would not

have phenotypic consequences in mitosis because arm cohesion would be sufficient to hold the chromatids together. This redundancy is not provided solely by ORD, a Drosophila protein required for arm cohesion in meiosis, because flies lacking both *mei-S332* and *ord* have demonstrated no abnormalities in somatic mitoses (S. E. Bickel, D. P. M., C. Lai, and T.O.-W, manuscript submitted).

Alternatively, it is possible that MEI-S332 does play a non-redundant role in mitosis, but it is required only in response to perturbations of the cell cycle. For example, if it were necessary for a cell to delay the onset of anaphase, persistence of MEI-S332 at the centromeric regions could in principle restrain the sister chromatids from separating. The discovery of a mitotic phenotype, under any conditions, would greatly enhance our understanding of the mitotic function of MEI-S332.

Meiotic cytology

Because MEI-S332 localizes to centromeres throughout meiosis until anaphase II, we were able to use it as a tool to examine meiotic chromosome morphology. In metaphase I arrested oocytes, the two caps of MEI-S332-GFP demonstrate that the centromeric regions of homologs are closest to the spindle poles during metaphase I, as would be anticipated if homologs are connected by chiasmata on the chromosome arms. Again using MEI-S332 to identify centromeres and chromosome orientation, we were able to infer an order of events after anaphase I. We found that in Drosophila as in Xenopus and other organisms, oocyte chromosomes do not decondense between the two meiotic divisions (Murray and Hunt, 1993), in contrast to spermatocyte chromosomes that do decondense between the divisions (Cenci et al. 1994).

Conclusions

Our finding that MEI-S332 is present on both meiotic and mitotic chromosomes reinforces the idea that meiosis and mitosis are highly conserved processes, even at the molecular level. In both types of divisions, it is localized to the centromeric regions of sister chromatids aligned on a bipolar spindle, and it is no longer present on the sister chromatids when they segregate from one another in anaphase. The function of MEI-S332 is essential during meiosis and not mitosis probably because of the meiosis-specific requirement that sister chromatids remain attached in the centromeric region during the first meiotic cell division. It is ironic that MEI-S332 is now implicated in mitosis, since if it had a strong

mitotic phenotype, lethality would have hindered the genetic and cytological analyses that defined its role in sister-chromatid cohesion. Our findings indicate that the analysis of meiosis will lead to a deeper understanding of chromosome segregation mechanisms in general.

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Chapter Five
The Chromosome Cohesion Protein MEI-S332 Interacts with the Microtubule-Associated Kinase LK6

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* AWP initiated the two-hybrid screen and supervised as CAF completed it, isolated the interacting plasmids, retested them, and tested their specificity. AWP retested the specificity of the LK6 library plasmid and its interactions with N- and C-terminal parts of MEI-S332, and performed the genetic tests, the GST pull-down assays, and the cytology.

INTRODUCTION

Meiotic and mitotic nuclear divisions require coordination of the spindle apparatus and the chromosomes. In most animal cells, spindles are organized by the centrosomes, which duplicate in interphase, migrate to opposite sides of the nucleus in prophase, and act to stabilize the minus-ends of microtubules as the spindle assembles during prometaphase (for review, see Kellogg et al., 1994). In mitotic cells, this "centrosome cycle" is tightly coupled with the cell cycle. In Drosophila oocytes, centrosomes are not present during the meiotic divisions (Theurkauf and Hawley, 1992), but gamma-tubulin, a protein usually associated with the centrosome, is required for proper meiotic spindle structure (Tavosanis et al., 1997).

During M-phase the spindle microtubule fibers emanating from the centrosomes are believed to grow and shrink more rapidly than in interphase, effectively dissolving the interphase microtubule network (Kirschner and Mitchison, 1986). These dynamic microtubules are stabilized when they are captured by kinetochores of mitotic chromosomes, and it is through such chromosome-microtubule interactions that a bipolar spindle is built (reviewed by (Waters and Salmon, 1997). However, in order for a microtubule/kinetochore interaction to be stable, it appears that the attachment must be under tension. In experiments on meiosis I spermatocytes from grasshoppers, it was determined that if the kinetochores on a chromosome pair were attached to microtubules emanating from the same pole, the chromosome pair "reoriented," that is, lost its microtubule attachments and made new ones. When kinetochores from the chromosome pair were attached to microtubules from opposite poles in a bipolar orientation, then the attachments were stabilized (Nicklas, 1967). Similarly, in laser ablation studies on mammalian mitotic cells, it was found that single unpaired kinetochores often made unstable attachments (Khodjakov et al., 1997). Direct micromanipulation experiments in grasshopper spermatocytes have demonstrated that mechanical tension across the chromosome is required for kinetochore/microtubule stabilization (Nicklas and Koch, 1969).

The spindle assembly checkpoint is a surveillance mechanism that cells use to make sure that all chromosomes are properly attached to the spindle and that the spindle is properly assembled, before attempting anaphase. In yeast, the *mad* and *bub* mutants, identified because they do not arrest in response to microtubule-depolymerizing drugs, are involved in the spindle-assembly checkpoint (for review see Murray, 1995). Several spindle assembly checkpoint genes have vertebrate homologs that are located at the centromeric regions of chromosomes (Straight,

1997). Even without perturbations, the spindle assembly checkpoint can sense the presence of a chromosome not attached to the spindle, and will delay anaphase until the attachment is made (Li and Nicklas, 1995; Rieder et al., 1994).

To achieve bipolar orientation on the spindle, sister chromatids need to maintain their cohesion to each other. If sister-chromatid cohesion is not maintained, the chromatids separate precociously before anaphase, and bipolar tension cannot be achieved to stabilize the spindle attachment. In mitosis, sister chromatids are attached to each other along their arms and at centromeres, and all cohesion is lost at anaphase. In meiosis, cohesion is more complex. Sister chromatids are attached along their arms and at centromeres until anaphase I, when homologous chromosomes segregate and arm cohesion is released. Cohesion must be maintained at the centromeric region until anaphase II, when the sister chromatids separate (for reviews see Bickel and Orr-Weaver, 1996; Miyazaki and Orr-Weaver, 1994; Moore and Orr-Weaver, 1998). In Drosophila, this centromeric cohesion requires the *mei-S332* gene, as *mei-S332* mutants cannot faithfully segregate their chromosomes in meiosis II (Kerrebrock et al., 1992). In both males and females, MEI-S332 protein is localized at the centromeric region until anaphase II, suggesting that MEI-S332 may act to physically hold the chromatids together until it disappears and they separate (Kerrebrock et al., 1995; Moore et al., 1998).

It is widely believed that in order for anaphase to proceed in mitotic cells, an anaphase inhibitor must be degraded by the anaphase-promoting complex (APC), a ubiquitin-ligase also responsible for the degradation of cyclin B (Holloway et al., 1993; Irniger et al., 1995; King et al., 1995). The Pds1p protein of *S. cerevisiae*, and the Cut2p protein of *S. pombe* appear to be inhibitors of anaphase that are degraded in an APC-dependent manner (Cohen-Fix et al., 1996; Funabiki et al., 1996). Yet since neither of these proteins localizes to the mitotic chromosomes, it is likely that they are only one step in an anaphase-promoting pathway. The *S. cerevisiae* protein Ssc1p/Mcd1p, like MEI-S332, is required for sister-chromatid cohesion and localizes to chromosomes. However, Scc1p is not degraded at the initiation of anaphase (Guacci et al., 1997; Michaelis et al., 1997)

Previously, we have shown that MEI-S332 is not degraded at the metaphase/anaphase II transition in oocytes (Moore et al., 1998), suggesting that some other regulatory event may inactivate it at this time. To identify potential regulators and other interacting proteins, we undertook a two-hybrid screen to find proteins that interact with MEI-S332. We report here that LK6, a microtubule-associated kinase, physically interacts with MEI-S332. Based on similarities between the

phenotypes of LK6 overexpression (Kidd and Raff, 1997) and *mei-S332* loss-of-function (this study), we suggest that LK6 negatively regulates MEI-S332.

MATERIALS AND METHODS

Two-hybrid screening

We used the two-hybrid *S. cerevisiae* strains, vectors, and methods developed by the Brent lab. These reagents and methods, along with all media recipes, are fully described in (Golemis et al., 1997). The *mei-S332* H12 cDNA was cloned into the pEG202 bait vector which fused the lexA DNA-binding domain to the N-terminus of MEI-S332. This high-copy vector contains the selectable *HIS3* gene. In order to utilize the *Eco*RI site in pEG202, the H12 cDNA (*Bam*HI - *Dra*I fragment; Kerrebrock et al., 1995) was recloned into pBluescript KS (Stratagene) at *Bam*HI and filled-in *Spe*I sites, thus removing the *Eco*RI linker sites in the original H12 construct and forming the KS/H12 #5 construct. KS/H12 #5 was digested with *Eco*RI and *Not*I and the H12 insert was recovered, then cloned into the *Eco*RI and *Not*I sites of pEG202, forming the full-length MEI-S332 bait construct. To make the N-terminal bait construct, the *Eco*RI - *Bgl*II N-terminal third of *mei-S332* was isolated from KS/H12#5 and cloned into the *Eco*RI and *Bam*HI sites in pEG202. The C-terminal MEI-S332 bait construct was made by isolating the *Bgl*II - *Dra*I fragment from the original H12 cDNA construct and cloning it into the *Bam*H1 and filled-in *Xho*I sites in pEG202.

Immunoblotting ensured that the full-length MEI-S332 protein was expressed from this construct in yeast. Protein extracts from strain EGY48 with and without pEG202 were made by resuspending cells in 50 mM Tris pH 7.5, 1 mM EDTA, 50 mM DTT, 1 mM PMSF, 0.5 mM TPCK, 0.025 mM TLCK, and 2 mg/ml pepstatin, and adding a volume of glass beads equal to the cell pellet. The cells were broken on a vibrax at 4° for 5 minutes. The extract was boiled in 1/2 volume of 4X SDS, and then the glass beads and insoluble material were pelleted. 40 μg of protein per lane was run on a 10% 37:1 acrylamide gel. The separated proteins were transferred to Immobilon (Millipore), blocked in TBS with 2% BSA and 5% dry milk, and incubated with anti-MEI-S332 peptide antibody (Moore et al., 1998) at a 1:30 dilution in TBS overnight at room temperature. After washing, the blot was incubated with 1:7500 alkaline phosphatase-coupled anti-rabbit secondary antibodies (Promega) for 30 min. at room temperature, and developed with the BCIP/NBT color substrate (Promega).

We used the RFLY3 Drosophila ovary acid fusion library made by Russ Finley in June 1993 (Finley et al., 1996). This library was poly-A selected and inserted into pJG4-5, a high copy plasmid which contains the selectable *TRP1* gene and expresses in-frame cDNAs fused at their N-terminus to an SV40 nuclear localization signal and the B42 acid blob transcription activation domain. Before amplification, the original

library complexity was 3.2×10^6. The average insert size was about 800 bp, with most inserts between 300 to 1500 bp (Golemis et al., 1997).

The strain EGY48 contains a genomic *LEU2* reporter gene with the upstream sequence replaced by LexA operator sites. The other reporter gene, *lacZ*, is encoded by the pSH18-34 plasmid.

To transform the library plasmids into the test strain, 400 ml of EGY48 harboring the pEG202/H12 bait and pSH18-34 *lacZ* reporter plasmids were grown to an OD_{600} of 1.0. After washing, the cells were resuspended in 2 ml LiOAc/TE (10 mM Tris HCl pH 7.5, 1 mM EDTA, 100 mM LiOAc, pH 7.5). This suspension was divided into 10 tubes of 100 µl each, and to each tube was added 2 µg of library DNA and 100 µg of sheared salmon sperm carrier DNA. DMSO was added to 10%, and 600 µl of 40% PEG 4000 in LiOAc/TE was added and each tube was incubated at 30° for 30 minutes. Cells were heat-shocked at 42° for 40 min.

We selected for interactors directly. Transformed yeast were diluted 10-fold into galactose -ura -his -trp media and incubated with shaking for 4 h at 30°. This media maintained selection for the bait, reporter, and library plasmids while inducing library-fusion protein expression. Cells were washed in water and plated onto galactose/raffinose (gal/raf) -ura -his -trp -leu plates. These plates selected for library fusion proteins that could activate transcription of the *LEU2* gene. Transformant colonies were picked after up to 10 days of growth at 30°, and transferred to gal/raf Xgal -ura -his -trp plates (Xgal: 5-Bromo-4-Chloro-3-Indolyl-β-D-galactopyranoside, from Diagnostic Chemicals) to examine their ability to activate β-galactosidase. Most colonies failed to turn blue; those that did were struck for single colonies on gal/raf -leu plates, then patched onto master glucose plates and replica plated onto the four diagnostic plates for testing interactors. (The diagnostic plates were glucose -ura -his -trp -leu; galactose/raffinose -ura -his -trp -leu; glucose Xgal -ura -his -trp; galactose/raffinose Xgal -ura -his -trp.) Strains that grew on -leu plates and turned blue on Xgal plates only in the presence of galactose were classified as light or dark blue interactors and stored at -80°C.

Library plasmids were recovered from yeast in a variation of the technique of Hoffman and Winston (1987). Plasmids were electrotransformed into *E. coli*; since these yeast harbored three plasmids all selectable only by ampicillin, minipreps and restriction digests were performed to identify bacterial lines with the library plasmid. Restriction digests were performed with *Eco*RI and *Xho*I, and in some cases further diagnostic digests with *Hind*III, *Eco*RV and *Nde*I confirmed that two independently isolated library plasmids had the same identity.

Library plasmids encoding dark blue interactors were retested by transforming them back into EGY48 with pEG202/H12 and pSH18-34, and checked for galactose-dependent growth on -leu media and blue substrate production on Xgal by replica plating. To test for specificity, these plasmids were also transformed into EGY48 strains containing the *lacZ* reporter plasmid and the bait plasmids pRFHM1, encoding a fragment of BICOID (Golemis et al., 1997), pEG202/PLU, pEG202/N-ORD or pEG202/C-ORD (L. M. Y. and Sharon Bickel, unpublished data). These strains were replica plated to the four diagnostic plates to determine whether the library proteins interacted in a galactose-dependent manner with the non-specific baits.

Library inserts were sequenced using the BCO1 primer (CCAGCCTCTTGCTGAGTGGAGATG) to sequence from the 5' end, and the BC02 primer (GACAAGCCGACAACCTTGATTGGAG) to sequence from the 3' end. Automated sequencing was performed at the Whitehead Sequencing Facility by Liuda Ziagra. Homology to known genes was found by using the BLAST homology search program.

GST pull-down assays

pGEX1 vectors containing in-frame fusions with *LK6* cDNA fragments were a gift from Jordan Raff. These vectors were transformed into BL21(DE3) *E. coli*, and fresh transformants were grown at 30° in LB + 100 µg/ml ampicillin to an OD_{600} of 1.0. IPTG was added to a concentration of 0.1 mM, and the cells were grown at room temperature for an additional 4 hours. Cell pellets were suspended in 5 volumes PBS (14 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2, 138 mM NaCl, 2.7 mM KCl) containing 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, and 0.1% Tween 20, and lysozyme was added to 200 µg/ml. Lysates were incubated at room temperature for 30 min., then gently sonicated on ice until the lysate was no longer viscous but still thick. KCl was added to 0.3 M and DTT added to 15 mM. Lysates were dialyzed against 30 volumes of PBS + 0.3 M KCl + 1 mM DTT, three times for 1 hour each at 4°. The extract was spun at high speed for one hour to remove insoluble material, then stored at -80°C in aliquots. To purify GST-fusions, extract was added to 20 µl glutathione-agarose beads (Sigma) and incubated at room temperature for 30 minutes. The GST-fusion protein concentration in these extracts was estimated to be 240 µg/ml for the GST moiety alone (in the pGEX.2T vector), 10 µg/ml of GST-PCR, 8 µg/ml of GST-LK6, and <0.1 µg/ml of GST-3.0. Enough extract was added to the beads to recover about 5µg of fusion protein, except for GST-3.0, where we added enough extract to recover

about 0.5 μg of undegraded fusion protein. Beads were washed 2X in either Tris-KCl (20 mM Tris 7.5, 100 mM KCl, 1 mM DTT, 0.5 mM EDTA, and 0.5% Triton X-100) or 0.5X Tris-KCl. 300 μl of either Tris-KCl or 0.5X Tris-KCl and 100μl of *E. coli* extract (a blocking agent), prepared as above and suspended in either Tris-KCl or 0.5X Tris-KCl, were added to the GST-bound beads with 5 μl of in vitro translated MEI-S332 protein.

For in vitro translation, the *mei-S332* cDNA from KS/H12#5 was cloned into pCITE 4a (Novogen) at the *Bam*HI and *Not*I sites. Protein was produced with the TnT T7 Quick Coupled Transcription/Translation System (Promega), following the manufacturer's instructions. 5 μl of this reaction was added to the GST-bound beads with the *E. coli* extract and buffer. Binding proceeded at room temperature for 30 minutes, then the beads were washed 3X in the appropriate buffer. The beads were boiled in 25 μl of 4X Laemmli sample buffer, and the eluate was loaded onto a 16% 200:1 acrylamide gel. Gels were stained with Coomassie blue to evaluate GST-fusion protein binding, and autoradiograms were taken with a Fuji phosphorimager. Quantitation was performed with the MacBas software program.

Drosophila genetics

All *mei-S332* alleles have been previously described (Kerrebrock et al., 1992) except 9 and 10 (Sharon Bickel, Tracy Tang, and T. O.-W., unpublished data). All *mei-S332* homozygotes designated in this study were actually transheterozygous, i.e. homozygous for *mei-S332* and heterozygous for most of the rest of the chromosome. Transheterozygous flies were constructed from *mei-S332* allele-containing chromosomes that have been outcrossed on both arms to two different chromosomes (see Kerrebrock et al., 1992).

All meiotic missegregation tests are variations on those of Zitron and Hawley (1989), as described in Kerrebrock, et al. (1992), although in our tests not all of the sex chromosomes were marked. This allowed recovery of fewer classes of exceptional progeny (progeny from missegregation events). For the non-complementation tests, males of genotype $+/y^{+}Y$; *mei-S332/+; Df(3R)TO-7/+* were crossed to compound *XX*, $y^{2}\ su(w^{a})\ w^{a}$. Phenotypic progeny classes from this cross are:

sex chromosomes in male gametes		sex chromosomes in female gametes: compound *XX*, y^2 *su(w^a) w^a*	0
normal	*X*	lethal	y^+ w^+ male
	Y	y^+ su(w^a) w^a female	lethal
exceptional	0	y^2 su(w^a) w^a female	lethal
	XX(Y)	lethal	y^+ w^+ female

In this cross, meiosis II missegregation events, leading to *XX* and nullo male gametes, can be observed as phenotypically distinct progeny.

For female non-complementation tests, females of genotype *+/y ; mei-S332/+ ; Df(3R)TO-7/+* were crossed to compound *XY, v f B* males. Phenotypic progeny classes from this cross are:

sex chromosomes in female gametes		sex chromosomes in male gametes: compound *XY*, *v f B*	0
normal	*X*	B female	B^+ male
exceptional	0	v f B male	lethal
	XX	lethal	B^+ female

In this cross, meiosis I or II missegregation events, leading to *XX* and nullo female gametes, were observed as phenotypically distinct progeny. Because only half of the exceptional progeny were viable, whereas all the normal progeny were viable, to calculate the % missegregation it is assumed that an equal number of inviable exceptional progeny exist: % missegregation = 2(exceptional progeny)/(total actually progeny + exceptional progeny).

Similar progeny classes were observed from the cross to measure enhancement or suppression of the *mei-S332* mutant phenotype in female meiosis: *y w* females with the appropriate second and third chromosomes (see Table 5-4) were crossed to compound *XY, v f B* males. % missegregation was calculated as for female non-complementation tests above. For tests to measure enhancement or suppression of the *mei-S332* mutant phenotype in male meiosis, *y w* males with appropriate second and third chromosomes were crossed to compound *XX*, y^2 *su(w^a) w^a* females.

In this test, the only class of exceptional progeny that could be identified were *XX* exceptions, and so it was not possible to calculate the % missegregation. The enhancement/suppression tests were performed in 3 - 7 vials containing the same number of parents in each vial. Statistics were calculated by the Statview program, and were based on comparing the distributions of the % exceptional progeny across all the vials of the same cross.

Non-complementation tests were first performed with a large deletion that removes the *LK6* locus (*Df (3R) M-Kx1* , 86C1; 87B5, data not shown). The deletion used throughout this study was *Df (3R) TO-7* (86F1-4; 86F8-12). Our experiments demonstrated that the *TO-7* chromosome also contains a w^+ insertion. For the enhancer/suppressor tests and viability tests, the *P[w+]* insertion was *P[w+, T-014 31-9A, cosmid 26]* (from Sharon Bickel, Bickel et al., 1996).

Cytology

Our first efforts at cytological examination were hampered by the presence of commensal parasites, probably Wolbachia, visible by chromatin staining as dots scattered around the nuclei and spindle poles. These parasites are known to disrupt mitosis in progeny from some crosses (Glover et al., 1990). To eliminate these parasites, stocks were treated with 0.025% Tetracycline for one generation and then allowed to recover for one generation before use.

Embryos were fixed either in 37% formaldehyde for 3 min., shaken in methanol/heptane to crack off the vitelline membrane and permeabilize the embryos, and incubated in methanol for two hours at room temperature; or the formaldehyde step was omitted and embryos were fixed in methanol alone. They were incubated with 1μg/ml propidium iodide (Sigma) to stain DNA, and bound with anti-β-tubulin antibodies (Amersham) to visualize microtubules, as previously described by Page and Orr-Weaver (1996).

RESULTS

Two-hybrid screening with MEI-S332

Genetic studies have demonstrated that *mei-S332* is required for cohesion of sister chromatids in meiosis (Kerrebrock et al., 1992), and more recently in mitosis (Heidi LeBlanc, Tracy Tang, Jim Wu, and T. O.-W., manuscript submitted). Cytological studies showed that MEI-S332 is localized to the centromeric regions of sister chromatids before they separate at anaphase, in both meiosis and in mitosis. Yet nothing is known about how MEI-S332 is regulated or with what it physically interacts. In order to identify regulators and effectors of MEI-S332, we conducted a two-hybrid screen to identify genes encoding proteins that bind to MEI-S332.

We used the method developed by Gyuris and Golemis of the Brent laboratory (Gyuris et al., 1993), a variant on the method of Fields and Song (1989). We introduced the *mei-S332* coding sequence into the pEG202 "bait" vector, which fuses the lexA DNA-binding domain to the N-terminus of MEI-S332. This plasmid was transformed into the *S. cerevisiae* strain EGY48. To confirm that the LexA-MEI-S332 fusion bait was properly expressed, we immunoblotted extracts from yeast that do and do not contain the MEI-S332-expressing pEG202 and observed a band from the MEI-S332 expressing strain of about the expected size (data not shown).

In the EGY48 + pSH18-34 strain, a plasmid-encoded lexA moiety binds to lexA binding sites constructed in the promoter regions of the endogenous *LEU2* gene and of a plasmid-encoded *lacZ* gene. This strain was used to screen a Drosophila ovary cDNA library inserted into the yeast vector pJG4-5 (Finley et al., 1996). In the presence of galactose, this vector expresses in-frame cDNAs fused at the N-terminus to a nuclear localization signal and an acidic transcriptional activation domain. cDNAs that encode proteins binding to MEI-S332 will bring the transcription activation domain in contact with the lexA DNA binding domain, and activate transcription of the reporter genes *LEU2* and *lacZ* in a galactose-dependent manner. An ovary library was screened because at the time these studies were initiated, it was clear that MEI-S332 functioned and was expressed in ovaries (Kerrebrock et al., 1992; Kerrebrock et al., 1995).

To screen for interactors, MEI-S332 bait-containing yeast were transformed with the library plasmids, and after a brief recovery plated directly on galactose-containing plates lacking leucine to select for interactors. The *LEU2* reporter gene is considered to be sensitive to weaker interactions than the *lacZ* reporter gene. Transformants growing on leu- media were then tested on galactose-containing X-gal

plates to determine qualitatively how much β-galactosidase activity was present. Intense blue color indicated a possible strong interaction, while a weaker blue color indicated a potentially weaker interaction. All putative interactors were retested to be sure the activation of the reporter gene was galactose-dependent.

We screened 1.7 x 10^7 clones, almost 6 times the complexity of the pre-amplified library (see figure 5-1 for an overview of screening process). Based on the intensity of the blue β-galactosidase (β-gal) reaction product, we identified 72 interacting constructs. Of these, nine stained intensely blue and 63 stained more lightly (see table 5-1 for a list of interactors in each category). Because the intensity of the β-gal reaction product can correlate with the strength of the interaction, we focused our efforts on the nine dark blue interactors.

To ensure that the transcription of the reporter genes was caused by the cDNA-containing plasmid and not by a background chromosomal mutation, each of the nine interacting clones was retested in a new EGY48 *lacZ*-containing host. All nine clones retested positively. These nine clones represented six independent cDNAs, as determined by restriction mapping and sequencing (data not shown).

As a secondary screen, each interactor was tested against non-related proteins expressed as lexA-tagged bait to examine the specificity of the interaction with MEI-S332. These interactions were assayed as β-gal blue reaction product on galactose/Xgal pates. The library cDNA clones were transformed into EGY48 containing two Drosophila proteins as baits that are unrelated to MEI-S332 in structure and function: the anterior pattern morphogen BICOID (BCD) and the inhibitor of DNA replication PLUTONIUM (PLU). Both of these proteins have been found to be "sticky" in the two hybrid system, in that they interact with many proteins (Golemis et al., 1997; L. M. Y., unpublished data). Four of the six classes of cDNA interacted strongly with one or both of these unrelated baits (see table 5-1), suggesting that these are not specific interactors of MEI-S332. These four were set aside. The two remaining classes, each identified twice in the screen, were 289/407 and 721/744. The 289/407 cDNA interacted very weakly with PLU as compared with MEI-S332, and not at all with BCD, and we retained it as a possible specific interactor. The 721/744 cDNA, later identified as the LK6 kinase, did not interact at all with PLU or BCD in our initial tests, and thus we retained it as a specific interactor. In later retests, we found weak interactions between LK6 and both PLU and BCD in about 1/5 tests.

At the same time, each of the interactors was tested against the Drosophila protein ORD (see table 5-1), which is required for sister-chromatid cohesion in

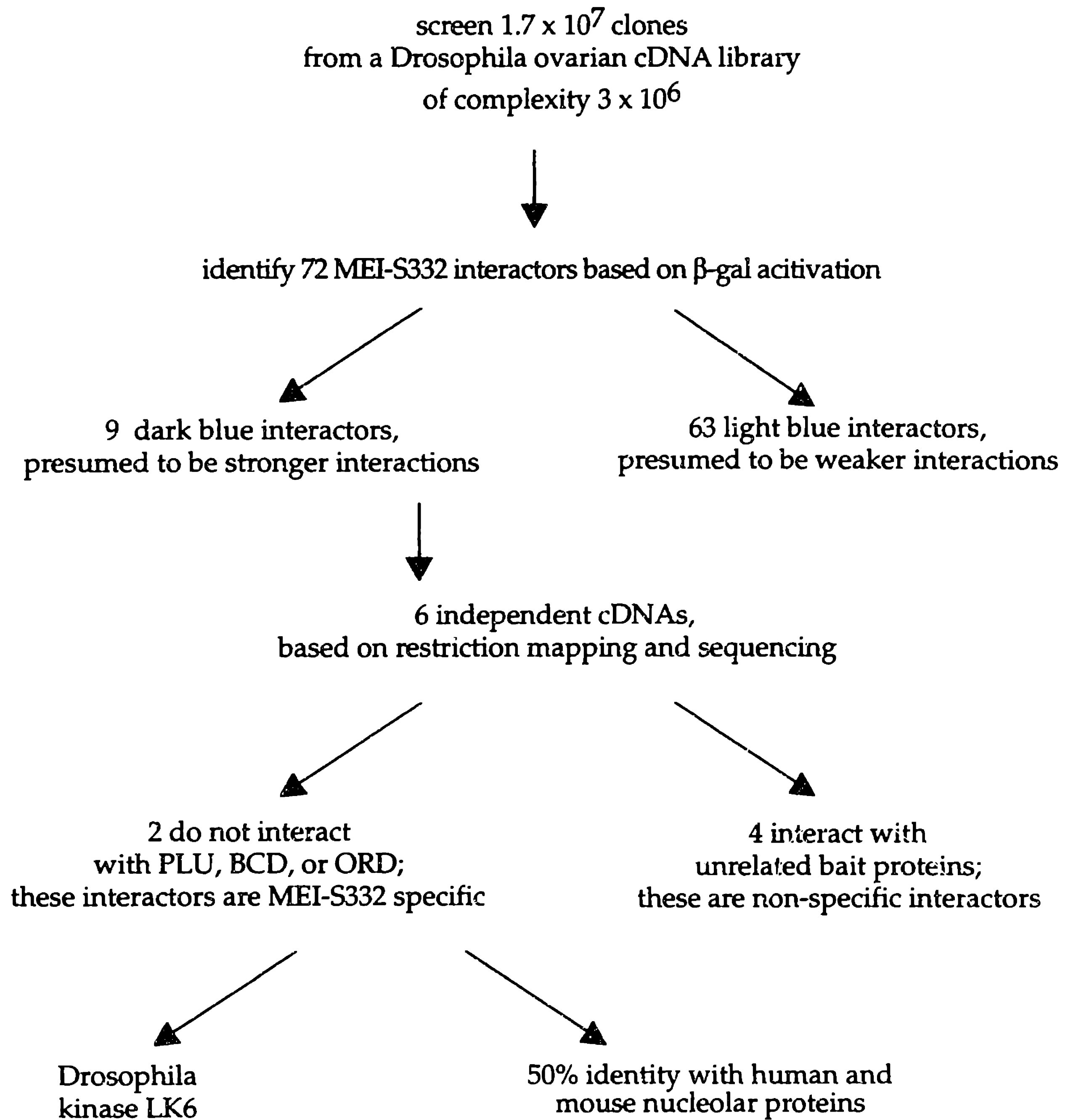


Figure 5-1. Overview of two-hybrid screening for MEI-S332 interactors.

Table 5-1. Two-hybrid interacting clones

I. Dark Interactors

library interactor number	strength of two-hybrid interaction with each bait					sequence identity
	MEI-S332 bait	BICOID bait	PLU bait	N-term. ORD bait	C-term. ORD bait	
721*	++	-	-	-	-	LK6 kinase
744*	++	-	-	-	-	
289*	+++	ND	+	+	+	nucleolar protein homolog
407*	+++	-	ND	-	-	
91	+++	++	-	+++	-	3′-UTR
598†	+++	+++	+++	-	-	ND
764†	++	+++	++	+	-	ND
462	++	++	++	+	-	ND
576	++	+	++	++	-	ND

* 721/744 and 289/407 are known to be the same clones from sequence data and restriction mapping.

† 598/764 are believed to be the same clone based on restriction mapping.

II. Light Interactors (isolation numbers)

1	18	123	132	169	213	214	217	220
223	224	227	228	235	237	254	255	258
260	261	263	269	270	276	281	287	293
296	298	306	313	316	317	318	324	349
354	357	365	373	375	404	416	439	444
449	455	544	580	608	614	617	620	634
637	640	648	685	700	709	713	723	779

meiosis and could conceivably interact with the same proteins as MEI-S332. Because the full-length ORD protein activates transcription of the reporter genes in the absence of galactose or prey protein, the full-length cDNA cannot be used in the two-hybrid assay (L. M. Y. and Sharon Bickel, unpublished data). To circumvent this problem, the ORD protein was divided into N-terminal and C-terminal fragments and cloned into the bait vector. Neither of these fragments activates transcription alone, and so can be used in the two-hybrid assay. Two-hybrid tests of the two MEI-S332-specific interactors demonstrated that neither interacts appreciably with either fragment of ORD (Table 5-1 and Fig. 5-2). Among the non-specific interactors, several interacted with ORD (Table 5-1).

Identities of specific interactors

The four independent clones that interacted specifically with MEI-S332 were sequenced, and it was confirmed that for both cDNAs we had independently isolated them each twice. Database searches found homologs for both cDNAs. The 289/407 cDNA was 50% identical across its full length to unpublished sequences described as nucleolar proteins from mouse and human (GenBank Accession numbers AF015308 and AF015039). The 721/744 cDNA was an exact match to the Drosophila serine/threonine kinase LK6 (Kidd and Raff, 1997). LK6 was first identified in biochemical assays for proteins that bound to taxol-stabilized microtubules. Kinase activity was predicted based on sequence homology, and autophosphorylation has been demonstrated. Its substrates are not known. Of eight antibodies raised against LK6, five do not recognize a specific localization pattern in embryos, and three localize LK6 to centrosomes of interphase and mitotic cells, although all eight recognize a band of the correct size on Western blots. Unfortunately, no Drosophila LK6 mutants exist.

Localizing the LK6 binding site in MEI-S332

Several recognizable motifs have been identified in the MEI-S332 protein sequence. MEI-S332 contains a predicted coiled-coil region, an acidic region, a basic region, and candidate PEST sequences (Kerrebrock et al., 1995). In order to learn what part of MEI-S332 binds to LK6, we tested N- and C- terminal fragments of the MEI-S332 protein in the two-hybrid system against the LK6 interacting fragment. The MEI-S332 fragments are schematized in Fig. 5-3A. Each of the MEI-S332 fragments, which together comprise the entire protein, were cloned into the bait vector pEG202. Unfortunately, the C-terminal fragment activated transcription in the

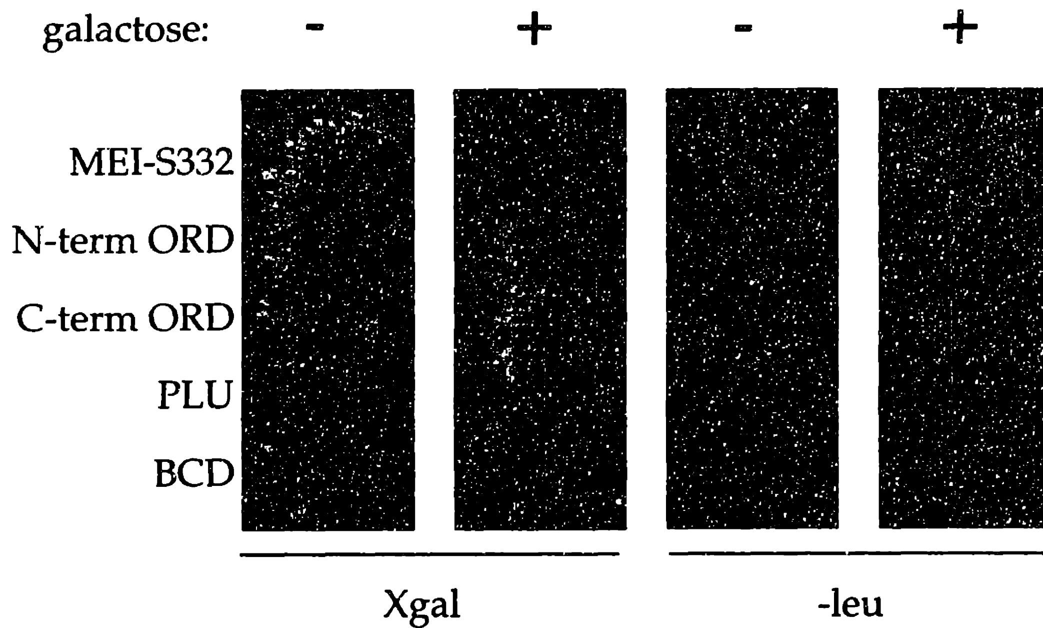
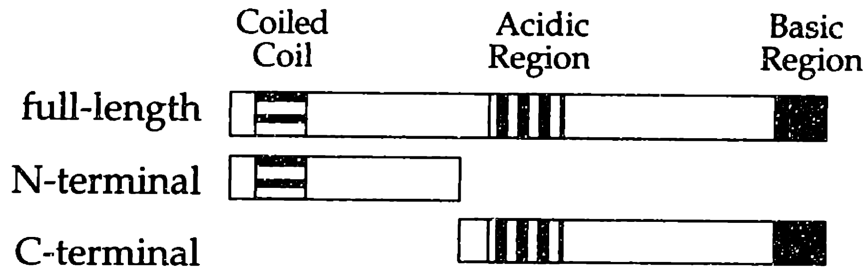


Figure 5-2. LK6 interacts specifically with MEI-S332 in the two-hybrid assay.

A plasmid encoding a galactose-inducible fragment of LK6 fused to an acid blob transcriptional activation domain (LK6 prey) was transformed into yeast strains containing plasmids with various genes fused to the LexA DNA binding domain (baits). LK6 consistently interacts with MEI-S332 but not with ORD (tested as N- and C- terminal fragments), PLU, or BCD, as assayed by transcriptional activation of *lacZ* or *LEU2* in the presence of galactose. Transformants were replica-plated from glucose plates onto appropriate test plates while maintaining selection for the bait, prey, and *lacZ* reporter plasmids.

A MEI-S332 baits:



B galactose: - + - +

full-length

N-terminal

C-terminal

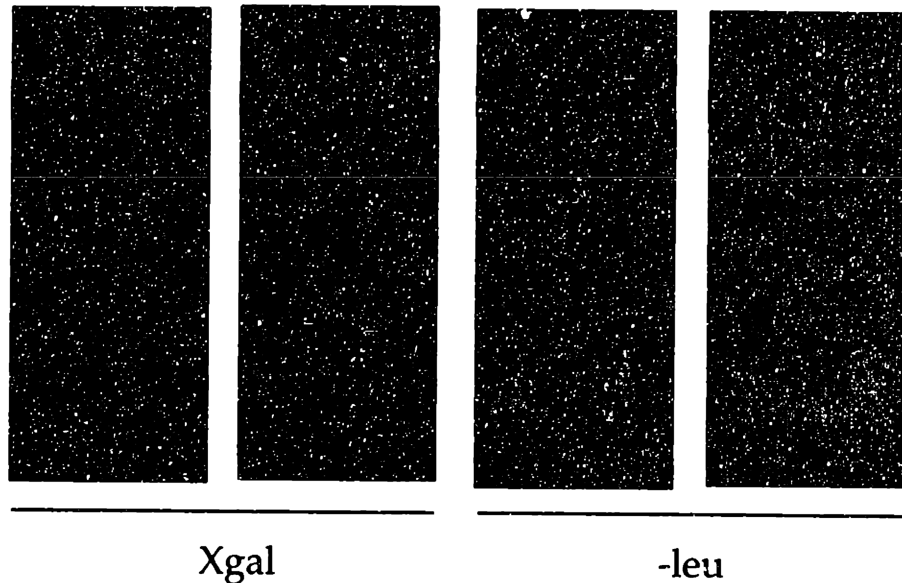


Figure 5-3. LK6 does not interact with MEI-S332 solely through the N-terminal third of MEI-S332 by the two-hybrid assay.

A. Full-length, N-terminal, or C-terminal fragments of MEI-S332 used as two-hybrid bait.

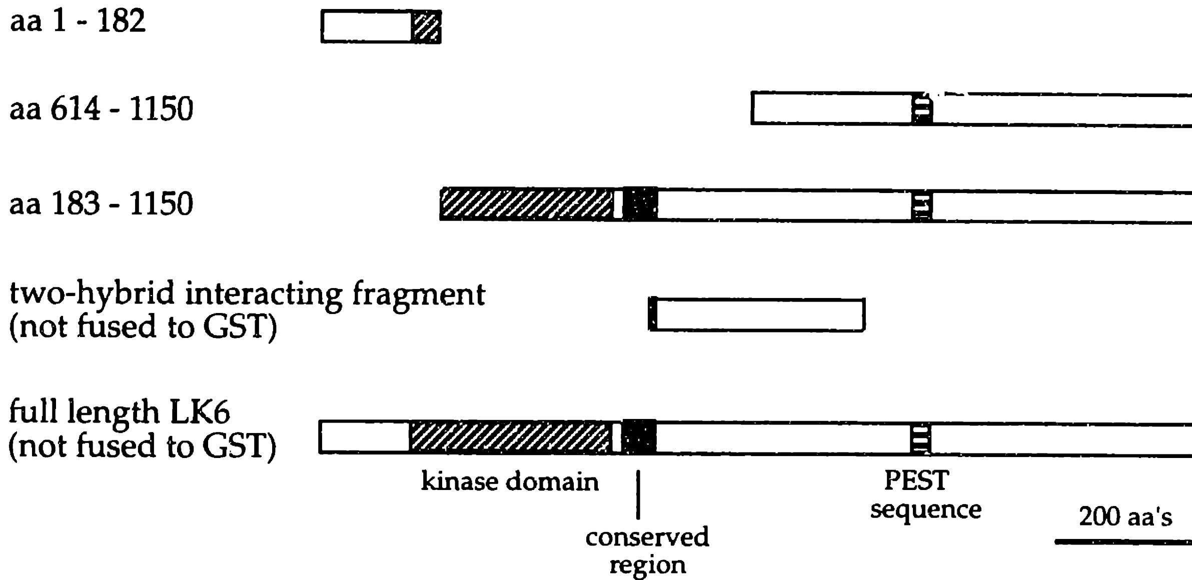
B. Bait plasmids encoding these fragments were transformed into yeast carrying the LK6 prey plasmid. The N-terminal fragment of MEI-S332 is not sufficient to activate the *lacZ* reporter construct, and it activates only weakly the more sensitive *LEU2* reporter construct in the presence of galactose. Because the C-terminal fragment of MEI-S332 can activate transcription alone in the absence of galactose, this assay cannot determine to what extent it interacts with LK6. Transformants were replica-plated from glucose plates onto appropriate test plates while maintaining selection for the bait, prey, and *lacZ* reporter plasmids.

absence of galactose-induced prey constructs (Fig. 5-3B), and so the results of the interaction test with LK6 were uninterpretable; in contrast, the N-terminal fragment was a usable two-hybrid bait. In galactose-containing media, the N-terminal fragment did not interact with LK6 sufficiently to activate the β-gal reporter gene, although they did interact sufficiently to allow slow growth without leucine via activation of the *LEU2* reporter (Fig. 5-3B). By both assays, the N-terminal fragment of MEI-S332 did not interact with LK6 as strongly as full-length MEI-S332. Thus it appears the interaction of MEI-S332 and LK6 is not mediated solely through the N-terminal piece of MEI-S332. Interestingly, it has been demonstrated that the N-terminus of MEI-S332 is sufficient for binding to full-length MEI-S332 in the two-hybrid screen (L. M. Y and T. O.-W., unpublished data), suggesting that the N-terminal third of MEI-S332 is sufficient to mediate homotypic interactions.

GST pull-down experiments

To confirm the interaction between MEI-S332 and LK6, we performed glutathione-S-transferase (GST) pull-down assays to determine whether these proteins interact in vitro. We chose this assay for two reasons. First, the full-length LK6 protein is extremely unstable in vivo with a half-life of about 20 minutes (Kidd and Raff, 1997). By using an in vitro assay we could avoid endogenous proteases. Secondly, it is possible that MEI-S332 is a substrate of the LK6 kinase, and a kinase/substrate complex would be unstable and difficult to identify. By testing fragments of LK6 that do not contain the complete kinase domain, we hoped to stabilize any transient protein complexes.

We tested three different fragments of the LK6 protein fused to GST, all gifts from Jordan Raff. These fragments are diagrammed in Fig. 5-4A. Comparing the fragments to the region of LK6 that was identified in the two-hybrid screen, we expected that the fragment containing amino acids 183-1150 would bind to MEI-S332 and that the fragment containing amino acids 1-182 would not bind to MEI-S332. In vitro translated MEI-S332 was incubated with the LK6-GST fragments and the GST moiety alone as a control, each immobilized on glutathione beads, in the presence of non-specific blocking proteins. This interaction was tested in two different salt conditions. As expected, the 183-1150 fragment, which contains the entire region identified by the two-hybrid screen, was able to bind MEI-S332 under both conditions (Fig. 5-4B). The fragment containing amino acids 614-1150, which contains only about half of the region identified by two-hybrid screening, was not able to bind to MEI-S332. The lack of binding suggests that the MEI-S332 binding



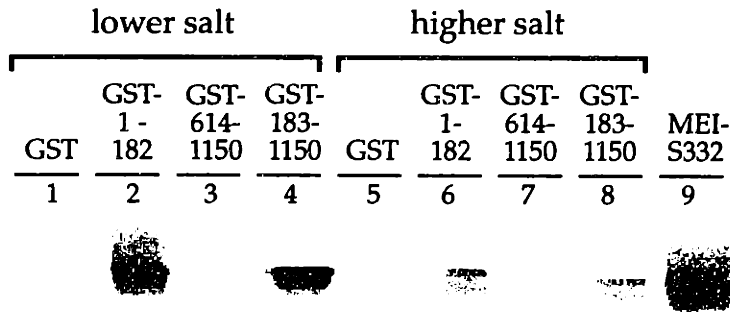


Fig. 5-4. MEI-S332 binds to LK6 in vitro.

GST-pull down assays were performed between GST-fusions of LK6 fragments and MEI-S332 radiolabeled protein.
A) The fragments of LK6 fused to GST (gifts from Jordan Raff) are shown with the full-length LK6 protein and the two-hybrid interacting piece for comparison. The fragments are designated by the amino acids contained in them.
B) MEI-S332 was translated and radiolabeled with 35S in vitro, then incubated with GST-fusions of LK6 immobilized on glutathione-agarose beads. After washing, the proteins were eluted in boiling SDS sample buffer and separated by electrophoresis and exposed on a phosphorimager. MEI-S332 binds to both the PCR and 3.0 fragments of the LK6 protein as shown in lanes 2, 4, 6, and 8; it does not bind to either GST alone or the "LK6" fragment of LK6. Lane 9 is the pre-bound in vitro translation reaction.

site is not fully contained within the 614-1150 fragment. These results confirm that MEI-S332 and LK6 physically interact.

To our surprise, the small fragment containing amino acids 1-182, which has no overlap with the region identified by two-hybrid screening, was able to bind to MEI-S332 at levels comparable to the 183-1150 fragment (Fig. 5-4B). This interaction suggests that LK6 has two separate regions each capable of binding MEI-S332. Although it is possible that LK6 has a single binding region that runs through both fragments, such a model requires that the MEI-S332 binding region of LK6 include the kinase domain. Alternatively, it is possible that two distant regions of the linear protein lie near each other in the folded protein and together bind MEI-S332. Finally, it is conceivable that LK6 can bind two different molecules of MEI-S332 at the same time. This is an especially interesting idea since MEI-S332 is believed to have a homotypic interaction.

Genetic tests of *mei-S332* and *LK6* interaction

Although the LK6 and MEI-S332 proteins can interact in the context of yeast cells and in vitro, it is important to establish that these two proteins interact in a functional manner in Drosophila. To investigate the functional significance of this interaction, we examined the effect of a heterozygous deletion of *LK6* (*Df (3R) TO-7*, hereafter referred to as *ΔLK6*) in various *mei-S332* mutant backgrounds. Because no mutants have been reported in *LK6*, it was not possible to examine interactions with homozygotes of *LK6*. We performed three separate tests with *LK6* heterozygotes: second-site noncomplementation, viability, and enhancement or suppression of the *mei-S332* homozygous mutant phenotype.

Ten *mei-S332* alleles have been characterized (Kerrebrock et al., 1992; Tracy Tang, Sharon Bickel, and T. O.-W., unpublished data). All are recessive, and homozygotes of all alleles tested are viable. *mei-S332* homozygous males and females have defects in the segregation of the sister chromatids during meiosis resulting in aneuploid progeny. The phenotype is assayed by examining the segregation of marked sex chromosomes. Missegregation is observed as the presence of marked exceptional progeny (those resulting from missegregation; see materials and methods for details).

Our first strategy to examine genetic interactions between *mei-S332* and *LK6* was to perform second-site noncomplementation tests. This assay has been used to detect interactions between gene products, and it is often allele-specific (Hays et al., 1989; Regan and Fuller, 1988; Stearns and Botstein, 1988). Because the test is rela-

tively simple, it was feasible to test all ten alleles of *mei-S332*. The test consisted of examining progeny from the double heterozygotes *mei-S332/+; ΔLK6/+* to determine whether the sex chromosomes were properly segregated. Small scale preliminary tests using a very large deletion that removed dozens of loci (*Df (3R) M-Kx1*) suggested that mei-*S332*^{8} failed to complement *ΔLK6; mei-S332* alleles 1,2,4, and 7 gave intermediate results (data not shown). These five *mei-S332* alleles were tested in larger numbers against the smaller *LK6* deletion *Df(3R)TO-7* for failure to complement in both males and females. For a negative control *mei-S332*^{3}*/+ ; ΔLK6/+* heterozygotes were tested, because this strain had no missegregation in the previous tests and the chromosomal background of these flies would be the most similar to the chromosomal background of the possible non-complementers. About 1000 progeny were scored for each test.

The results are shown in Table 5-2. In our tests no missegregation was observed in the *mei-S332*^{3}*/+ ; ΔLK6/+* double heterozygote negative control. In even larger tests it has been reported that wild-type and *mei-S332* heterozygous females have a missegregation rate of 0.05-0.1% (Kerrebrock et al., 1992). Against this background, a rate of 0.53% missegregation in *mei-S332*^{4}*/+ ; ΔLK6/+* double heterozygotes may indicate that these loci fail to complement in females. In males, wild-type and *mei-S332* heterozygotes missegregate their sex chromosomes at a frequency of about 0.1%, and exceptional progeny are usually nullo sperm which contain neither an *X* or a *Y* chromosome (Kerrebrock et al., 1992). Against this background, the rate of 0.33% missegregation in *mei-S332*^{8}*/+ ; ΔLK6/+* double heterozygotes, which includes more *XX* exceptions than nullo exceptions, may be significant. It appears then that *mei-S332*^{4} may fail to complement *ΔLK6* in females and *mei-S332*^{8} may fail to complement ΔLK6 in males; however, we are cautious about the interpretation of these results because the absolute numbers of exceptional progeny are small. Additionally, other genes are expected to be absent or disrupted in the deletion chromosome used to study *ΔLK6* , and thus it cannot be ruled out that missegregation is caused by the failure of another locus to complement *mei-S332*.

The second genetic test that we undertook to examine a functional interaction was an examination of the viability of *mei-S332* homozygotes in the presence of *ΔLK6*. The MEI-S332 protein is known to localize to the centromeric regions of mitotic chromosomes until anaphase in many mitotic tissues (Moore et al., 1998; Heidi LeBlanc, Tracy Tang, Jim Wu, and T. O.-W., manuscript submitted), and loss-of-function and overexpression analysis demonstrates that it functions at the centromere to maintain sister-chromatid cohesion (Heidi LeBlanc, Tracy Tang, Jim

Table 5-2.
Genetic interactions between *mei-S332* and *ΔLK6*
I: Complementation data

progeny from $\frac{\textit{mei-S332}}{+}$; $\frac{\Delta \textit{LK6}}{+}$ †

	allele	total progeny	XX exceptional progeny	nullo-*X*,*Y* exceptional progeny	% missegregation (adjusted*)
female tests	*mei-S332*[1]	1028	1	0	0.19%
	mei-S332[2]	1023	0	1	0.20%
	mei-S332[3]	1035	0	0	0
	mei-S332[4]	1126	1	2	0.53%
	mei-S332[7]	1088	0	0	0
	mei-S332[8]	1088	0	1	0.18%
male tests	*mei-S332*[1]	951	0	0	0
	mei-S332[2]	1042	0	1	0.10%
	mei-S332[3]	826	0	0	0
	mei-S332[4]	1023	0	0	0
	mei-S332[7]	916	0	1	0.11%
	mei-S332[8]	915	2	1	0.33%

† *ΔLK6* is *Df (3R) TO-7.*

* % missegration is adjusted to account for classes of exceptional progeny in the female tests that are inviable and so cannot be recovered. (See Materials and Methods.)

Wu, and T. O.-W., manuscript submitted). Nevertheless, careful examination of *mei-S332* homozygous mutants has demonstrated that mutants survive to adulthood equally well as their heterozygous siblings (Kerrebrock et al., 1992; Heidi LeBlanc, Tracy Tang, Jim Wu, and T. O.-W., manuscript submitted); thus *mei-S332* is not required for viability. We asked whether *mei-S332* homozygote viability was decreased when the gene dose of *LK6* was reduced by half, by comparing the survival of *mei-S332* homozygotes with an *LK6* deletion to that of their siblings without the deletion. The results are shown in Table 5-3. For the three alleles of *mei-S332* we tested, alleles *2*, *6*, and *8*, we recovered approximately equal numbers of homozygous siblings with and without the deletion. Thus, the loss of one copy of *LK6* does not decrease the viability of *mei-S332* homozygotes.

The third genetic test of the interaction of *mei-S332* and *LK6* was to ask whether a heterozygous deletion of *LK6* enhanced or suppressed the *mei-S332* mutant phenotype, i.e., does meiotic chromosome missegregation get worse or better in *mei-S332* homozygotes with only one copy of *LK6?* To perform this test, we compared chromosome missegregation rates in *mei-S332* male and female homozygotes without the deletion to their homozygous siblings with the heterozygous deletion. By comparing siblings we were able to control more closely for background effects. These tests were performed in several small batches (vials) and from these we calculated the average missegregation rate. Breaking up the results into small batches allowed us to take into account the variation within each genotype when comparing the segregation rates. We tested *mei-S332* alleles *2*, *6*, and *8* because these alleles displayed a range of weak, intermediate, and strong phenotypes in the two sexes such that we believed it would be possible to identify changes by enhancement or suppression. The results are shown in Table 5-4. In all mutants, the variation within each genotype from vial to vial was so great that no significant difference could be detected between homozygotes with and without the deletion.

New *mei-S332* embryo phenotypes

It is problematic to envision how *mei-S332* and *LK6* could interact together because they have not been localized to the same place. MEI-S332 localizes to centromeres and holds sister chromatids together. LK6 localizes throughout the cytoplasm and possibly at centrosomes. Overexpression of the protein causes centrosomes to become uncoupled from spindles in embryos, suggesting that the normal function of LK6 is at centrosomes. Nonetheless, these localization patterns do not rule out the possibility that the proteins interact. To gain insight into how

Table 5-3.
Genetic interactions between *mei-S332* and ΔLK6 †
II: Viablity data

yw ; $\frac{mei\text{-}S332}{+}$; $\frac{\Delta LK6}{+}$ X $\frac{yw}{\rightarrow}$; $\frac{mei\text{-}S332}{+}$; $\frac{P[w^+]}{+}$

allele	*mei-S332* ; $\frac{\Delta LK6}{+}$ (#)	*mei-S332* ; $\frac{P[w^+]}{+}$ (#)
mei-S332^2	99	108
mei-S332^6	101	95
mei-S332^8	114	111

† *ΔLK6* is *Df(3R) TO-7*.

Table 5-4.

Genetic interactions between *mei-S332* and *ΔLK6* †

III: Enhancer/suppressor data

I. Female Tests

genotype of females tested	total progeny	% missegregation (adjusted*)	% exceptional progeny per vial (not adjusted)	P value
mei-S332^{2}; *P/+*	126	39.5%	12%, 21%, 36%	0.349
mei-S332^{2}; *ΔLK6/+*	425	45.4%	19%, 26%, 29%, 30%, 31%, 40%	
mei-S332^{6}; *P/+*	668	10.7%	3.3%, 5.8%, 6.6%, 7.5%	0.138
mei-S332^{6}; *ΔLK6/+*	935	19.0%	3.9%, 5.9%, 7.9%, 13%, 17%, 19%	
mei-S332^{8}; *P/+*	899	4.6%	0.5%, 1.1%, 1.1%, 2.7%, 7.9%	0.125
mei-S332^{8}; *ΔLK6/+*	1084	1.1%	0, 0, 0.6%, 0.6%, 0.9%, 1.2%	

II. Male Tests

genotype of males tested	total progeny	total *XX* exceptions/ total progeny (%)	*XX* exceptional progeny per vial (%)	P value
mei-S332^{2}; *P/+*	1094	6.3%	3.9%, 5.0%, 5.1%, 5.6%, 6.5%, 8.0%, 10%	0.407
mei-S332^{2}; *ΔLK6/+*	1040	5.0%	0.6%, 2.1%, 2.1%, 6.8%, 7.4%, 10.2%	
mei-S332^{6}; *P/+*	950	0.53%	0, 0, 0, 0.6%, 0.7%, 0.8%, 1.4%	0.410
mei-S332^{6}; *ΔLK6/+*	860	0.81%	0, 0, 0.6%, 0.7%, 1.4%, 2.4%	
mei-S332^{8}; *P/+*	986	6.6%	4.4%, 4.8%, 5.6%, 6.0%, 6.4%, 7.4%, 11%	0.179
mei-S332^{8}; *ΔLK6/+*	1009	4.8%	2.7%, 3.1%,4.0%, 4.6%, 4.9%, 7.1%, 8.0%	

† *ΔLK6* is *Df(3R)TO-7*

* For females, % missegregation is adjusted to account for inviable classes of progeny that cannot be recovered, see Materials and Methods.

these proteins might function together, we reexamined the role of MEI-S332 in the early mitotic divisions in embryos.

Previously, two types of studies had examined the role of MEI-S332 in the embryonic mitotic divisions and had concluded that the protein was not required there. In viability studies, it had been determined that *mei-S332* homozygotes survive equally well as their heterozygous siblings to adulthood (Kerrebrock et al., 1992; Heidi LeBlanc, Tracy Tang, Jim Wu, and T. O.-W., manuscript submitted). In a second type of experiment, embryos were studied cytologically for defects in mitosis (A. Kerrebrock and T. O.-W., unpublished data). This study examined the later embryonic divisions on the surface of the embryo, after about cycle 10, because these divisions are easier to visualize. Yet embryos after cycle 7 have acquired an "editing" mechanism to discard defective nuclei that are aneuploid or asynchronously cycling by depositing them into the center of the embryo where the yolk nuclei reside (Sullivan et al., 1993). By studying only later embryos that have "edited" out defective nuclei, this previous study was unable to observe early mitotic defects and less likely to observe defects overall. In both these studies, if *mei-S332* were required very early in embryogenesis, its role could have been missed. For our re-examination, we chose to examine embryos before cycle 7 because these embryos would still have all of their nuclei whether or not they were defective.

Embryos between the ages of 0 and 2 hours old were collected from *mei-S332*^{7} homozygous mutant mothers; *mei-S332*^{7} encodes a prematurely truncated protein and is one of the strongest *mei-S332* alleles (Kerrebrock et al., 1992; Kerrebrock et al., 1995). These embryos were fixed with either formaldehyde or methanol, and stained with propidium iodide to visualize DNA and anti-tubulin antibodies to visualize microtubules. We were surprised to find that a significant fraction of the embryos had defective spindle or chromosome morphology or asynchronous nuclei (Fig. 5-5). We observed spindles where the spindle poles appeared partially detached from the spindle body, spindles without spindle poles, and free tubulin asters. With the DNA staining, we observed chromosomes that were overcondensed, chromosomes that had come apart from the main mass of chromosomes, chromosomes irregularly placed on the spindle, and asynchronous nuclei. These aberrant spindles and nuclei were observed in both methanol- and formaldehyde-fixed embryos.

We were concerned that the mitotic defects we observed might be secondary effects of a meiotic missegregation event. That is, if the maternal pronucleus was aneuploid, that might be sufficient to cause defects in nuclear or spindle morphology. To control for pronuclear aneuploidy, we compared eggs laid by *mei-S332*

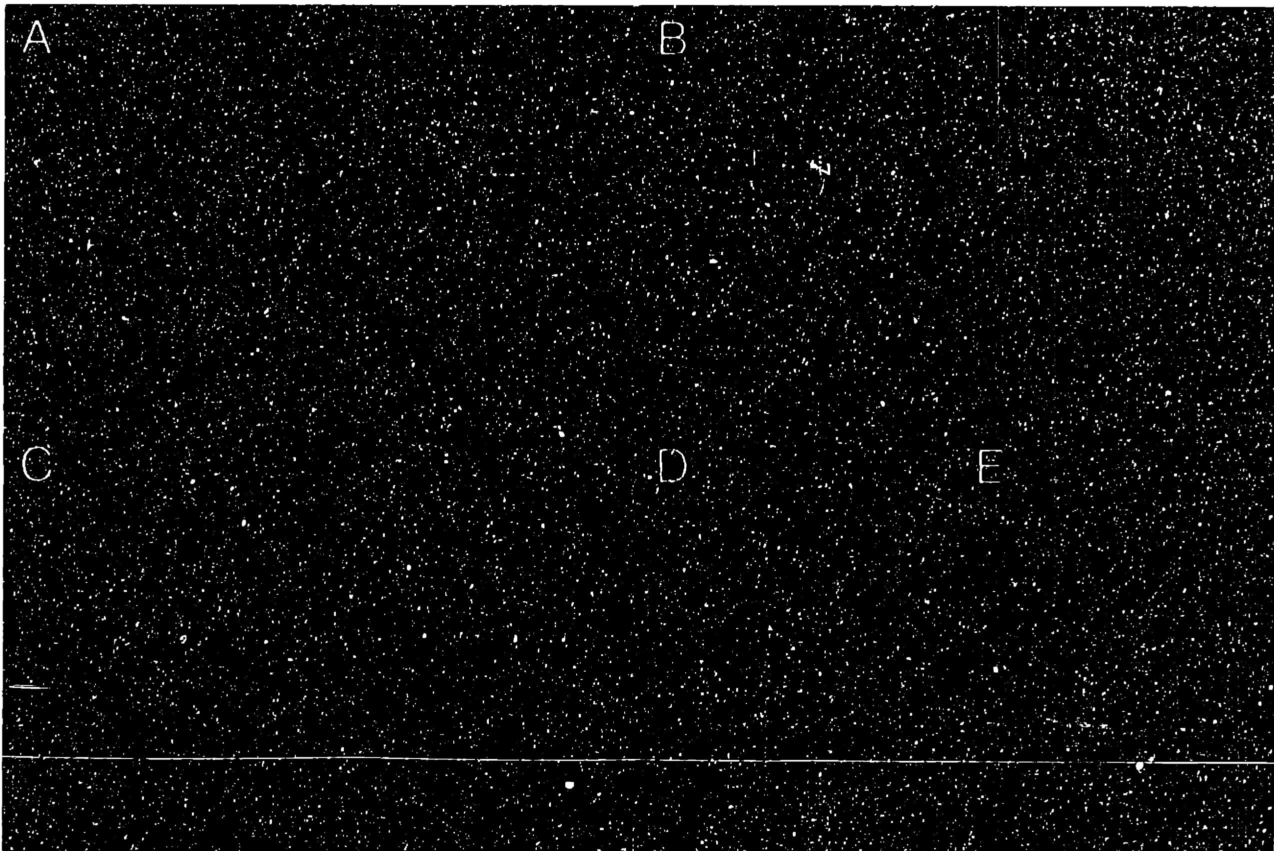


Figure 5-5. Embryos from *mei-S332* mutant mothers have defects at their spindle poles.

Embryos were fixed in methanol, stained for DNA and tubulin, and imaged by confocal microscopy. A-D are from *mei-S332*^{7} mutant mutant mothers and E is from a wild-type mother. Stocks were treated with tetracycline to avoid artifacts from commensal parasites. Many focal planes are projected into one plane in these images.
A-C) are embryos in cycle 2.

A) The spindle asters appear to have separated from both mitotic spindles.

B) Extra asters of tubulin are observed, suggesting that there are more centrosomes than spindles. The poles associated with the spindles appear to be further from the body of the spindle than in wild type (compare to E).

C) One spindle has lost its poles and appears round while the poles of the other spindle are unusually far from the body.

D) A spindle from a mutant embryo where one chromosome has separated from the others, although it is still attached to spindle fibers.

E) A spindle from a wild-type embryo in cycle 4 fixed and stained at the same time as the mutants shown. The poles are closer to the body than in the mutants.

mothers mated to wild-type males with eggs laid by wild-type mothers crossed to *mei-S332* males. Because *mei-S332*^{7} causes similar missegregation frequencies in male and female homozygotes (Kerrebrock et al., 1992), in the reciprocal crosses the same frequency of embryos will be aneuploid. Although the aneuploidy will be held constant, the levels of MEI-S332 protein in early embryos will reflect the genotype of the mother: in one case the embryos will lack MEI-S332 and in the other case they will have a wild-type complement of the protein. To establish quantitatively whether the mitotic phenotypes are caused by aneuploidy from meiotic missegregation or from a mitotic defect itself, we counted the number of embryos with defective spindles and nuclei in each of the reciprocal crosses and in a control wild-type cross (Table 5-5). There were virtually no abnormalities in the embryos laid by wild-type mothers (either crossed to *mei-S332*^{7} males or wild-type males) whereas almost a quarter of the embryos from *mei-S332*^{7} mothers had defects. This percentage is likely to be an underestimate because many defects were not visible with the conventional epifluorescence microscopy we used for counting; with the confocal microscope many more embryos were observed to have spindle and centrosome defects. These defects demonstrate that MEI-S332 has a role in the early embryonic divisions distinct from its role in meiosis. It appeared that MEI-S332 was required for proper mitotic chromosome segregation (see Fig. 5-5E) and possibly proper regulation of the spindles in these early divisions.

Interestingly, we noticed that more of the embryos from *mei-S332* mothers were in the earliest cycles than were embryos from wild-type mothers (Table 5-5). It was not possible to conclude from the 0-2 hour experiment whether these earlier-staged embryos were progressing through the divisions at a slower pace, or whether this population coincidentally contained younger embryos. To discriminate between these two possibilities, we collected embryos for one hour from the same three crosses (the two reciprocal crosses and wild-type) and then aged them for one hour such that they were all 1-2 hours old. Embryos older than one hour should be just completing cycle 7, and this was the case for the embryos from the wild-type cross and those from wild-type mothers crossed to *mei-S332* fathers (Table 5-6). In contrast, about half of the embryos from *mei-S332* mothers were still in cycles 1-7; most of these were in cycles 4 or earlier. To ensure that this delay was not caused by a dominant modifier on the *mei-S332* chromosome, we examined mothers heterozygous for *mei-S332*^{7} and found fewer than 2% progressing through cycles 1-7 (data not shown). We conclude that *mei-S332* is required for timely and proper execution of the early embryonic mitotic divisions.

Table 5-5. *mei-S332* phenotypes in 0-2h old embryos

parental cross* creating embryos	spindle/DNA abnormalities (%)	undergoing cycles 1-4 (%)	number examined
☿ *mei-S332*^{7} X ♂ wild type	21%	50%	52
☿ wild type X ♂ *mei-S332*^{7}	0	21%	52
☿ wild type X ♂ wild type	2%	29%	65

* All stocks were treated with tetracycline to avoid artifacts caused by commensal parasites. Embryos were fixed and stained for DNA and tubulin.

Table 5-6. Delayed cycling in *mei-S332* embryos 1-2h old

parental cross* creating embryos	undergoing cycles 1-7 (%)	number examined
☿ *mei-S332*^{7} X ♂ wild type	48%	50
☿ wild type X ♂ *mei-S332*^{7}	5%	55
☿ wild type X ♂ wild type	6%	53

* All stocks were treated with tetracycline to avoid problems from commensal parasites. Embryos were fixed and stained for DNA and tubulin.

DISCUSSION

To identify regulators and effectors of sister-chromatid cohesion, we undertook a two-hybrid screen to find ovarian cDNAs that encode proteins that physically interact with MEI-S332. Of two specific interactors identified, one is the Drosophila microtubule-associated serine/threonine kinase LK6. We confirmed that this interaction takes place in vitro with a GST-pull-down experiment. Genetic experiments were unable to demonstrate convincingly that these proteins function together in vivo, although the genetic approach was hampered by the lack of a mutation in the *LK6* gene. We were intrigued, however, by the existence of a previously unsuspected role for MEI-S332 in the embryonic divisions. This phenotype is similar to the phenotype of *LK6* overexpressing embryos, and this similarity strengthens the argument that these two proteins function together in Drosophila. Additionally, recent preliminary results from anti-MEI-S332 immunoprecipitation experiments suggest that MEI-S332 and LK6 interact as a complex in Drosophila (Tracy Tang and T. O.-W., preliminary results).

Expected two-hybrid results

Two-hybrid screens have limitations. First, it is possible to pick up false positives because either the bait or prey protein is able to interact with many proteins, including those that have no biological relevance. We are confident that MEI-S332 does not behave in this indiscriminate manner because we picked up so few dark blue interactors in the screen. We also tried to eliminate library interactors with this characteristic by checking them against other bait proteins known to interact promiscuously; such secondary screens should rule out chaperonins and other highly sticky library proteins. Another limitation of the two-hybrid screen is that it is possible to miss biologically relevant interactors. For example, directed testing by the two-hybrid assay has demonstrated that MEI-S332 can bind to itself (L. M. Y. and T. O.-W., unpublished data), an interaction that has been confirmed by immunoprecipitation experiments (Tracy Tang and T. O.-W., unpublished data). Yet we did not recover MEI-S332 among our strong interactors. This can be explained in three ways. First, since the library was poly-A primed, it is possible that the reverse transcription did not proceed far enough to include the MEI-S332 homotypic interaction domain; this would make it impossible to identify MEI-S332 interactors among the cDNAs. Secondly, it is possible that MEI-S332 is not well represented in the cDNA library, either because the transcript itself is rare or because of over-amplification of the

library. Finally, it is possible that under our screening conditions, we would not have observed the self-interaction as strong interaction, and thus it may be among our uninvestigated weaker interactors. The weak interactors may be worth further analysis. Since it is clear that two-hybrid screening is only as good as the library that is screened, another MEI-S332 two-hybrid screen has been recently initiated using a more complex and less amplified library with longer cDNA inserts (C. Raymond, L. Dang, and T. O.-W, work in progress.)

The significance of LK6 and MEI-S332 interactions

The LK6 kinase was first identified biochemically by its ability to bind to taxol-stabilized microtubules (Kellogg et al., 1989). In immunofluorescence experiments with eight anti-LK6 antibodies, most antibodies did not demonstrate a specific localization pattern for LK6. The other three antibodies identified LK6 at the centrosomal regions of mitotic and interphase nuclei in Drosophila embryos (Kidd and Raff, 1997). No mutations in *LK6* exist, but *LK6* has been overexpressed from the strong ubiquitin promoter. Overexpressing flies have low viability. Of the embryos laid by survivors, roughly half do not develop, and the remainder have mitotic defects. In the developing embryos, it appears that the centrosome and cell cycles become uncoupled, as centrosomes pull away from nuclei in the cortical divisions (Kidd and Raff, 1997). One explanation for this phenotype is that LK6 is involved directly in centrosome function; we offer an alternative hypothesis based on its interaction with MEI-S332.

Embryos from LK6 overexpressing mothers and *mei-S332* loss-of-function mothers have similar phenotypes. Both have variable phenotypes that range from a complete failure of development to viable adult flies. Embryos of both genotypes have spindle poles that appear to disengage from the mitotic spindle, and free asters are observed without accompanying nuclei. This similarity of phenotype of loss-of-function and overexpression suggests that *mei-S332* and *LK6* are in a negative regulatory relationship. One simple model would have LK6 phosphorylate and thus inactivate MEI-S332; it is also conceivable that MEI-S332 might sequester and thus inactivate LK6.

How can these phenotypes be explained in terms of sister-chromatid cohesion? Our model of MEI-S332 and LK6 function in the early embryo is based on the idea that, as in meiosis and in larval tissues, MEI-S332 is required to hold sister-chromatids together; at all these times MEI-S332 localizes to prometaphase and metaphase chromosomes and is removed at anaphase. We think it likely that sister

chromatids lose cohesion before metaphase in embryos from *mei-S332* mutant mothers, and thus nuclei cannot make bipolar attachments to chromosomes and align them at the metaphase plate. Apparently, there is variability as to whether a particular nucleus is affected (see below). In nuclei that cannot align their chromosomes, the spindle assembly checkpoint arrests the cell cycle at metaphase. However, the centrosome cycle continues, causing separation of centrosomes from spindles in an anaphase-B like motion and centrosome duplication. This model accounts for why *mei-S332* mutants arrest at metaphase, show aberrant chromosome alignment on the spindle, and have centrosome duplication. The phenotype of embryos from *LK6* overexpressing mothers can be explained by LK6 constitutively inactivating MEI-S332 to such a degree that the *mei-S332* mutant phenotype is reproduced. Since embryos from *LK6* overexpressing mothers have significantly less viability than those from *mei-S332* mutant mothers, it is likely that LK6 also acts on other targets.

Previous studies have found that the centrosome cycle can continue in Drosophila embryos that have arrested the cell cycle. Centrosome behavior has been studied in embryos injected with DNA-replication inhibitor aphidicolin during the cortical divisions, around cycle 10. Studies on fixed embryos at varying times after injection first suggested that centrosomes could go through multiple rounds of replication in the absence of DNA replication (Raff and Glover, 1988). This question has been examined more recently in live embryos. Normal embryos were found to generate occasional free centrosomes as a result of a division error. Such normal free centrosomes continued to divide, often through at least two cycles. In contrast, centrosomes in embryos injected with aphidicolin only replicated once (Debec et al., 1996). It appears that in embryos from *mei-S332* mutant mothers, centrosomes replicate only once. It is not clear how to compare mutant embryos with injected embryos because they are in different stages of development, and because the mechanism of arrest is different. Embryos laid by mothers mutant for the genes *giant nuclei (gnu)*, *plutonium (plu)* or *pan gu (png)* overreplicate and fail to divide their nuclei; nonetheless, the centrosome cycle appears to continue faithfully for many rounds (Freeman et al., 1986; Shamanski and Orr-Weaver, 1991). In these embryos, however, it is not clear that the cell cycle is arrested.

The variability among embryos from *mei-S332* mutant mothers and *LK6* overexpressing mothers can be accounted for in several ways. First, experiments in mammalian culture cells have shown that single unattached kinetochores have highly variable fates. Sometimes they reorient, sometimes they remain attached to

poles, and sometimes the single kinetochore makes attachments to both poles and can congress to the metaphase plate (but not segregate at anaphase; Khodjakov et al., 1997). Separated sister chromatids might behave with similar variability in embryos from *mei-S332* mothers, allowing some nuclei to proceed while others cannot align their chromosomes. Secondly, it is possible that some mothers carry an enhancer or suppressor that is causing the variability in the phenotype, and this background modifier is not isogenic throughout the population. Finally, it is possible that the mutation studied, *mei-S332*^{7}, a truncation allele, is not a null allele. If this allele were able to function just around the threshold required during the early divisions, then stochastic processes might cause some embryos to cycle normally while others do not. Indeed, in separate experiments embryos from *mei-S332*$^7/\Delta$ mothers were found to have a slightly higher percentage of phenotypically abnormal embryos than the homozygotes studied in other experiments (data not shown).

The spindle assembly checkpoint appears to function in early embryos from some species and not from others. Mitotic extracts from Xenopus do not arrest the cell cycle in response to microtubule depolymerizing drugs until there is a high nuclei/cytoplasm ratio, suggesting that checkpoints are not active in the earliest cycles (Minshull et al., 1994). In the first division of sea urchin embryos, disruption of kinetochore attachments in 50% of the chromosomes was not sufficient to halt the cell cycle (Sluder et al., 1994). In contrast, microtubule depolymerizing drugs arrest nuclei at metaphase in Drosophila early embryos, suggesting that a spindle-assembly checkpoint is active (see Foe et al., 1993). This apparent difference in the existence of the spindle assembly checkpoint may be related to mechanisms of cell cycle regulation. Whereas in Xenopus embryos cdc2 activity cycles with the cell cycle, in Drosophila embryos cdc2 activity is constant in the early cycles (Edgar et al., 1994). To explain this constant activity level, it has been suggested that in early Drosophila embryos cyclin B is degraded locally around each nucleus but not throughout the embryo (Edgar et al., 1994). Local control of mitosis in Drosophila early embryos would allow each nucleus to make a local decision to arrest mitosis until the spindle is assembled and the chromosomes are attached.

If embryos from *mei-S332* mutant mothers display aberrant cell cycles, why has an effect not be observed in the viability studies? These studies compared siblings with identical maternal contributions of MEI-S332, and little or no zygotic MEI-S332 is produced until after the completion of most of the embryonic mitotic divisions (Heidi LeBlanc, Tracy Tang, Jim Wu, and T. O.-W., manuscript submitted). Therefore, even in studies comparing homozygous mutant and heterozygous sibling

offspring from *mei-S332* mutant mothers, the embryonic mitotic defects would be expected to be the same. Also, it has been observed that among embryos that lose 50% of their cells during the mitotic divisions, some can continue through development into adulthood (Sullivan et al., 1990). Similarly, in larval discs, another mitotically proliferating tissue, individuals can survive death in up to about 20% of cells (Baker et al., 1982). Thus, it appears that Drosophila is tolerant of variable numbers of surviving cells.

Although we present a model here that explains the function of LK6 on centrosomes as an indirect consequence of a spindle assembly checkpoint arrest, it remains possible that LK6 acts directly at centrosomes. However if LK6 acts directly at centrosomes, and it interacts with MEI-S332 which also has an effect on centrosomes, then there is a strong implication that MEI-S332 also acts directly at centrosomes. Since no other evidence exists for believing that MEI-S332 acts at centrosomes, we favor the hypothesis that the centrosome defects observed in embryos from *mei-S332* loss-of-function and *LK6* overexpressing mothers are a secondary consequence of sister-chromatid cohesion defects.

Future Experiments

More experiments will be needed before the functional significance of an interaction between MEI-S332 and LK6 can be understood. Because so much of the relationship between the two proteins and their inferred function is based on a previously unrecognized phenotype of *mei-S332*, confirmation of the spindle checkpoint arrest/centrosome defect in *mei-S332* embryos is very important. Injections of anti-MEI-S332 antibodies into wild-type embryos would be expected to reproduce the loss-of-function phenotype near the site of injection. This experiment could also address the puzzling question of penetrance, in that it could be determined whether all spindles near the injection site lose their centrosomes. An injection experiment also avoids any confusion in interpretation from the earlier meiotic missegregation phenotype.

Another important question is whether LK6 can phosphorylate MEI-S332. This experiment can be addressed in vitro with kinase assays, although the question of in vivo relevance is hard to address with an in vitro assay. Another approach is to immunoprecipitate MEI-S332 with anti-LK6 antibodies, and ask if LK6 can phosphorylate MEI-S332 in that complex. A less direct genetic approach would be to examine the status of MEI-S332 phosphorylation in extracts from flies overexpressing *LK6*. MEI-S332 migrates as a doublet on an SDS protein gel (Moore et al, 1998), and it

is possible that the relative intensity of these bands could change in the *LK6* overexpressors. Although conventional wisdom argues that changing the concentration of a kinase is unlikely to greatly change the rate of phosphorylation of the substrate, in this case it is already known that overexpression of the LK6 kinase has severe consequences for embryos. If it can be shown that MEI-S332 is a likely substrate for LK6, the next goal is to demonstrate the function of that phosphorylation event. One approach would be to identify the site of phosphorylation, and then introduce an inducible but non-LK6-phosphorylatable MEI-S332 into flies by transformation. If this mutant MEI-S332 cannot be inactivated, it would be expected to have a dominant phenotype upon induction, and cytological examination might reveal whether sister chromatids were unable to separate at the metaphase/anaphase transition in the second meiotic division and/or in mitosis.

Creating or identifying a mutant in LK6 would greatly aid in the analysis of LK6 function, but in the absence of such a mutation, it is worth carefully examining flies that overexpress a kinase-dead mutated LK6 for a phenotype (Kidd and Raff, 1997). This kinase-dead mutant might be expected to act in a dominant-negative fashion. Particularly interesting would be defects in meiotic or mitotic chromosome segregation, which would more directly implicate LK6 in regulating chromosome segregation and sister-chromatid cohesion.

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Afterword

I would like to reflect here on the work I have done, what work should be done next, and how this past and future research fits into larger questions about the study of meiosis. The studies described here reflect my curiosity about how the meiotic cell cycle works. I have focused my energies on meiosis in an animal oocyte model system because only oocytes have normal mechanisms to stop and restart the meiotic cell cycle for development and fertilization. These pauses and resumptions are superimposed onto an already radically modified cell cycle, where two nuclear divisions occur without replication.

Future Experimental Approaches

The work described in these chapters opens up several experimental approaches to the study of the meiotic cell cycle in Drosophila. Two new mutants, *grauzone* and *cortex*, appear to affect the oocyte meiotic cell cycle specifically, and these mutants may provide an entry point for the identification of related genes in genetic screens. The method of activating oocytes in vitro allows many eggs to enter the meiotic divisions at once, facilitating both cytological examination and also biochemical investigation. Finally, the identification of a kinase that may potentially be involved in sister-chromatid cohesion holds out the possibility of finding a pathway that connects the meiotic cell cycle with one of its downstream regulatory targets, cohesion of sister chromatids.

There are several experiments to be done given the results described here. I have shown that is likely that *grauzone* and *cortex* are involved in regulating the meiotic cell cycle, and not in regulating translation, as translation of new proteins is not required for progression of the meiotic cell cycle after metaphase I. The molecular identification of these genes is an essential next step. It would be extremely interesting if they had homology to known genes; if they encode novel products, localization studies of the gene products could give significant insight about their role in the progression of the meiotic cell cycle.

With the in vitro activation system, it is immediately possible to investigate the role of known mitotic proteins in the meiotic cell cycle. In principle it is possible to fix activated eggs in methanol and isolate small populations in the same stage of meiosis based on cytology. Extracts prepared from each small population can be probed for the presence of mitotic regulators, and for altered mobility of those regulators. For example, the mobilities of one active and three inactive forms of cdc2

have been carefully determined (Edgar et al., 1994); it should be possible by immunoblotting to determine what forms of cdc2 are present at which stages of meiosis and thus when cdc2 is active. Because translation is not required for the meiotic divisions, and because the condensed meiotic nucleus is not transcriptionally active, post-translational modifications are likely to be essential for the progression of the meiotic cell cycle. Modifications such as phosphorylation or degradation can often be visualized on immunoblots.

Biochemical experiments such as immunoblotting can complement an examination of mutant cytology. For example, several *cdc2* temperature sensitive alleles exist, and the cytology of eggs activated under non-permissive conditions might be informative. Such *cdc2* eggs could be compared to activated eggs mutant for *twine*, the cdc2-activating phosphatase, to see whether *cdc2* and *twine* are required for the same events. For mitotic genes without conditional alleles, such as the Drosophila MAPK *rolled*, it is possible instead to make germline clones so that heterozygous mothers make homozygous mutant oocytes. Activation of such oocytes could give information about the role of MAPK in the oocyte meiotic divisions in Drosophila.

The in vitro activation technique can also be used for answering specific questions. For example, one important question about the meiotic cell cycle is how it inhibits DNA replication between meiosis I and II. Three genes, *plutonium (plu), pan gu (png),* and *giant nuclei (gnu),* are known to be required to inhibit DNA replication in the earliest mitotic divisions of the embryo but not later in development. It is not clear whether the overreplication phenotype observed in these mutant embryos begins during meiosis. A meiotic requirement for these genes is an attractive hypothesis because it might be expected that specific genes would be required to inhibit replication between the meiotic divisions, genes that are not required later in development. Currently, in vitro activation experiments are in progress to determine whether these mutants can incorporate the nucleotide analog BrdU inappropriately during meiosis (Colleen Raymond, A. W. P. and Terry Orr-Weaver).

The relationship of LK6 and MEI-S332 is still unclear. It is difficult to plan any long-range studies until some basic questions are answered: does LK6 function in sister-chromatid cohesion or release? Does it function in meiosis? Does it phosphorylate MEI-S332 and if so for what purpose? The experiments at the end of chapter 5 address some of these questions, although the identification of an LK6 mutant would greatly aid in an assessment of its function.

Conservation and Homology in Meiosis

The biggest question facing researchers of the meiotic cell cycle is the question of evolutionary conservation. Drosophila is studied as a model organism, but can conclusions based on Drosophila meiosis be extrapolated to other organisms?

So far, there has been surprisingly little conservation among different animals with respect to the oocyte meiotic cell cycle. After the completion of recombination and prophase I, the animal kingdom varies widely in its meiotic arrest points and the phase of the cell cycle when fertilization takes place, as was seen in figure 1-1. Even among a group of evolutionarily close relatives such as mammals, dog oocytes are fertilized at a different meiotic arrest point than other mammals (John, 1990). At a molecular level, there is conservation between meiotic and mitotic cell cycle regulators, as predicted by the model that they evolved from a common cell cycle. Yet the particular meiotic functions of these regulators appears to differ between species. In mouse and Xenopus, cdc2 appears to be differently regulated between the divisions; in Drosophila, cdc2 may not even be required for the metaphase I arrest (AWP, preliminary data). Mos, the vertebrate meiosis-specific regulator, appears to have different functions in Xenopus and mouse. In both species it is required to arrest the oocyte at metaphase II, but only in Xenopus and not in mouse is it required to restart the meiotic cell cycle after the prophase arrest. No homologs of mos have been identified outside the vertebrate lineage. Homologs of the Drosophila meiosis-specific sister-chromatid cohesion proteins MEI-S332 and ORD have not been identified in any system to date.

This apparent lack of conservation may be just bad luck. It may turn out that with further study, deeper understandings, and more genomes completely sequenced, the molecular conservation will become obvious. But at this point, it is worth considering the less attractive alternative.

Is there a reason why meiosis and the meiotic cell cycle might be so highly variant when so many other processes are so highly conserved? First, the possibility cannot be excluded that meiosis evolved independently more than one time, although this does not seem to me to be a likely source of the tremendous variation observed in even closely related species. Divergence is especially evident later in the meiotic cell cycle, nearer to fertilization. It is believed that one of the selective pressures shaping animal evolution is reproductive competition between males and females (Chapman and Partridge, 1996; Morell, 1996; Rice, 1996). This intersexual competition is a potent force for evolutionary change, as each sex adapts and counter-adapts in highly specific ways, much like parasites and hosts. This coevolu-

tion can in principle create behavioral or physical barriers to fertilization, which in turn genetically isolates populations to form different species. It is plausible that the female meiotic cell cycle, especially as it relates to fertilization, could be a target of rapid evolutionary change as a result of intersexual conflict and coevolution. This could explain why species that are reasonably closely related could have so many differences in the meiotic cell cycle.

Perhaps a new set of expectations is required. Rather than search for families of widely conserved proteins, we need to focus on widely conserved biological problems. For example, replication must be repressed between meiosis I and II in order to achieve a reduction to a haploid genome. MAPK may be responsible in mice, cdc2 may be responsible in Xenopus, but the problem remains the same. Similarly, although the molecules that hold meiotic sister-chromatids together may turn out to be different between different organisms, the problem of maintaining and releasing sister-chromatid cohesion at the appropriate time in the two divisions remains the same. In all meiotic cell cycles, many regulatory events must be accomplished while the nucleus is condensed and transcriptionally inert during the two divisions; translational and post-translational modifications are likely to be dominant mechanisms of regulation during the meiotic cell cycle.

A search for common biological requirements, instead of a search for common biological mechanisms, will require an understanding of meiosis in many organisms. Such an understanding is valuable for pragmatic as well as intellectual reasons. Understanding the basic processes of meiosis has already shed light on the nature of chromosomal abnormalities in human conceptuses (Lamb et al., 1996) An understanding of the possibilities for regulating cell cycles generally may give insights into how cancerous cells can be misregulated. Knowledge of how the meiotic cell cycle is regulated in many species may open new opportunities for controlling our own fertility, a continuing need in this overpopulated world. For these practical reasons, study of the meiotic cell cycle should be pursued in Drosophila and other model organisms, even if molecular homology remains elusive. For intellectual reasons, Drosophila will likely be a leading model organism in the study of the meiotic cell cycle because the combination of genetics and cytology gives powerful insights.

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Appendix I: *png; cort* and *png; grau* Double Mutants†

Embryos laid by mothers homozygous mutant for *pan gu* overreplicate their DNA, leading to the appearance of giant polyploid nuclei. Strong alleles of *png* cause the DNA to replicate without nuclear division, and weak alleles allow a few nuclear divisions to proceed but the DNA still becomes overreplicated. As embryos age, the amount of DNA appears to increase in the giant nuclei. In young embryos it can be observed that the four meiotic products and the male pronucleus each overreplicates individually, but as the amount of DNA increases, these nuclei fuse. Because it is possible to see the four meiotic products in young embryos, it has been assumed that oocyte meiosis was completed normally before the initation of replication (Shamanski and Orr-Weaver, 1991).

To determine whether it was possible for *png* mutants to begin overreplication during meiosis, I constructed the double mutants *png; cortex* and *png; grauzone*. Eggs laid by either *grauzone (grau)* or *cortex (cort)* homozygous mutant mothers are arrested inappropriately in meiosis II, at either metaphase in younger embryos or at anaphase in older embryos. By constructing double mutants, I expected to hold the mutants in meiosis II and observe whether they could overreplicate their DNA.

In Table I-1, the different phenotypes of embryos from double mutant mothers are quantified, as are the phenotypes of sibling controls. Embryos 0-3 hours old from mothers homozygous mutant for both *png* and *cort* appeared to arrest in meiosis, as evidenced by the presence of condensed chromosomes, suggesting that *cort* is epistatic to *png*. In 0-2 hour old embryos from mothers mutant for both *png* and *grau*, however, the phenotype was less clear. Although some of the embryos (18%) arrested in meiosis with condensed chromosomes like *grau* mutants, the majority of the embryos (53%) had giant overreplicated nuclei characteristic of *png* mutants (Fig. I-1). Yet a third large class (28%) was not clearly either mutant phenotype. Many in this third class had numerous scattered, fragmented chromosomes that could be an intermediate between condensed chromosomes and replicating chromosomes (Fig. I-1B). To see if this class was in transition from a meiosis II arrest to overreplicating, we aged a three

† This data was included as "data not shown" in the publication by Lisa K. Elfring, J. Myles Axton, Douglas D. Fenger, Andrea W. Page, Janet Carminati, and Terry L. Orr-Weaver, "The Drosophila PLUTONIUM protein is a specialized cell cycle regulator required at the onset of development." Molecular Biology of the Cell 8, 583-593.

hour collection of embryos from *png; grau* mothers so that they were 3-6 hours old. More of these embryos displayed giant nuclei (65%) than did their younger cohorts, and fewer had the condensed chromosomes typical of a meiosis II arrest (7%). Thus it appears that embryos from *png; grau* mothers arrest in meiosis II as *grau* embryos do, but over time begin DNA overreplication typical of *png* mutants.

In conclusion, it appears that embryos from *png* mutant mothers can begin DNA replication during meiosis, since they replicate DNA in a *grau* mutant background, although it is still not clear when they begin replication in a wild-type background. It is possible that the *cort* gene product is necessary to support replication, whereas the *grau* gene product is not. An alternative interpretation is that *cort* acts at an earlier (but cytologically indistinguishable) step than *grau* to arrest meiosis II. A third alternative is that this allele of *grau* is leaky, allowing some gene function, whereas this allele of *cort* is not. The interpretation of this data is somewhat obscured by the knowledge that png^{3318}, the allele used in this study, is a weak allele of *png*.

Reference:

Shamanski, F., and T. Orr-Weaver. 1991. The Drosophila *plutonium* and *pan gu* genes regulate entry into S phase at fertilization. *Cell*. 66:1289-1300.

Table I-1.
Phenotypes of embryos from *png; cort* and *png; grau* double mutant mothers

age of embryos	genotype†	phenotype observed: giant nuclei	condensed chromosomes	other	developing*	number examined
0-3 h	*png; cort*	6%	89%	5%	0	63
0-3 h	*png/+; cort*	3%	95%	2%	0	98
0-3 h	*png; cort/+*	85%	0	15%	0	59
0-2 h	*png; grau*	53%	18%	28%	0	70
3-6 h	*png; grau*	65%	7%	28%	0	74
0-2 h	*png/+; grau*	6%	72%	22%	0	50
3-6 h	*png/+; grau*	0%	97%	0%	2%	58
0-2 h	*png; grau/+*	84%	0	16%	0	25
3-6 h	*png; grau/+*	92%	0	0	8%	25

† This indicates the genotype of the mothers. The phenotypes were assessed in embryos. The alleles used were png^{3318}, $grau^{QE70}$, and $cort^{QW55}$

* Developing 3-6 hour old embryos from *png; grau/+* mothers were determined to have lost one copy of the *png* allele (data not shown). If 8% of the *png* homozygotes are actually heterozygous in the *png; grau* siblings, then the levels of the overreplicating phenotype observed would be artificially low in 3-6 hour old embryos from double mutant *png; grau* mothers.

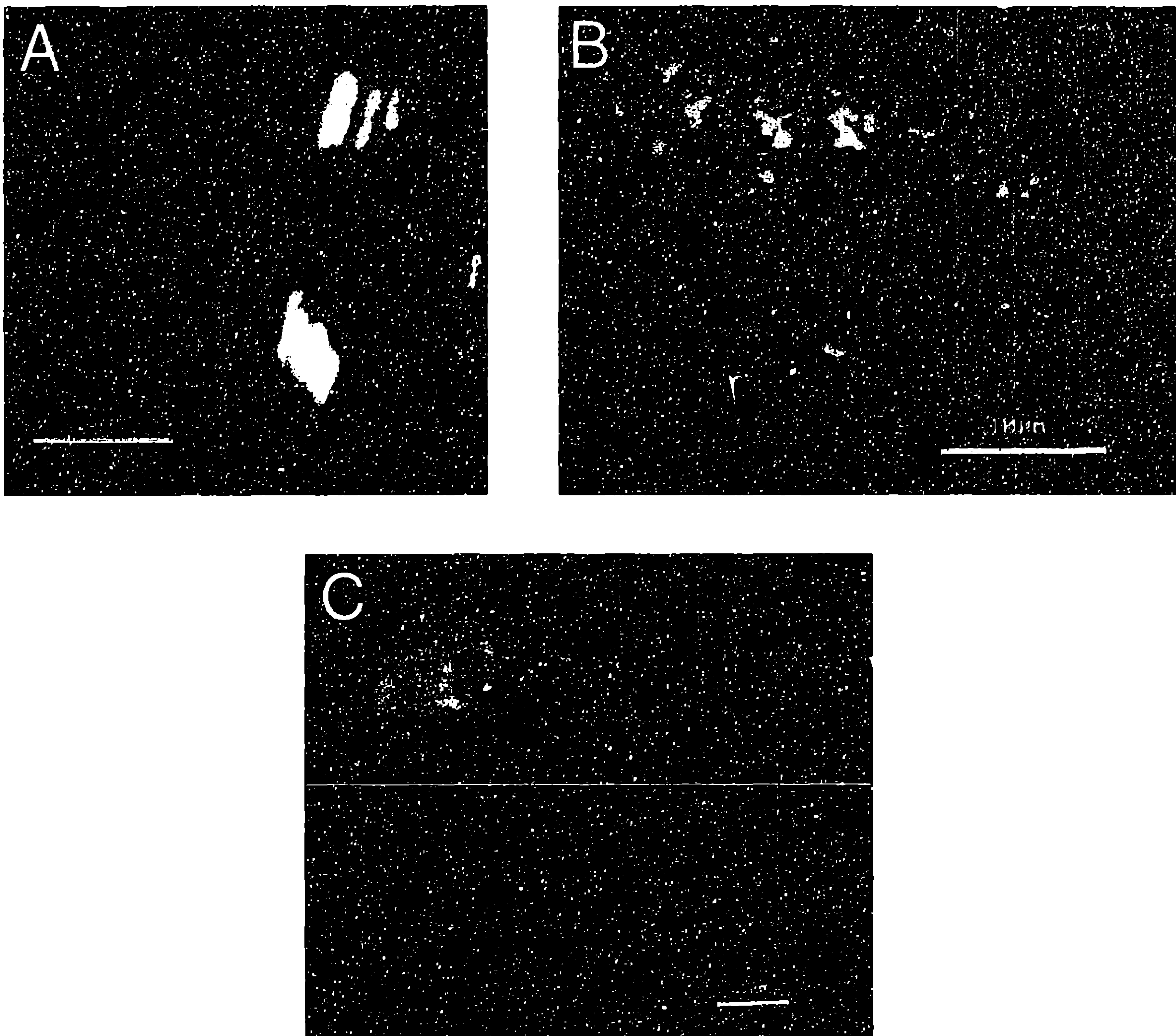


Figure I-1. Phenotypes of embryos from *png*^{3318}; *grau*^{QE70} mothers.
Chromatin is stained with propidium iodide.

A) Condensed chromosomes typical of the meiotic arrest in embryos from *grau* mothers.

B) Fragmented chromosomes, perhaps representing an intermediate stage between condensed meiotic chromosomes and nuclear overreplication.

C. Overreplicated DNA typical of embryos from *png* mothers. This image is a double exposure to show the size of the nucleus with respect to the embryo.

Appendix II:
Proteins Translated at Egg Activation in Eggs from *grauzone* and *cortex* Mothers

Mature Drosophila oocytes are activated at the end of oogenesis as they pass through the oviduct. Activation appears to cause a number of downstream events, among them release from the metaphase I meiotic arrest, a reorganization of the microtubule cytoskeleton, and the translation of new proteins. Eggs laid by *grauzone (grau)* and *cortex (cort)* homozygous mutant mothers arrest in an inappropriate meiosis II after aberrant chromosome segregation in meiosis I. These eggs are also unable to remodel their microtubule cytoskeleton, an event that usually occurs after activation of the oocyte (Page and Orr-Weaver, 1996). It has been reported that *grau* and *cort* eggs also cannot translate proteins that are usually translated after egg activation (Lieberfarb et al., 1996), suggesting that these mutants may be defective in another event downstream of egg activation.

We investigated the ability of mutant eggs to translate BICOID (BCD) protein and TOLL (TL) protein, both of which were believed to be translated only after egg activation in the oocyte (Driever and Nusslein-Volhard, 1988; Gay and Keith, 1992). Because antibodies no longer exist that recognize BCD protein on Western blots, we were constrained to examining BCD protein levels in whole mount embryo staining (see methods below). In embryos from *grau/+* heterozygous mothers, the expected anterior gradient of BCD protein was observed (Fig. II-1A). In contrast, in embryos from *grau* and *cort* homozygous mothers, no distinct pattern of BCD localization was observed (Fig. II-1B,C). Unfortunately, it is not possible to distinguish whether this is a disruption in the amount of BCD protein present or a disruption in its distribution.

To be able to more accurately examine protein levels, we examined whether TL protein was present in embryos from *grau* and *cort* mothers. Western blots of extracts prepared from *grau* and *cort* eggs demonstrated that TL was translated at levels comparable to heterozygous controls and wild type (Fig. II-2A). Surprised by this normal translation pattern, we examined whether indeed TL protein was first translated at egg activation. Extracts from mature stage 14 oocytes also had comparable levels of TL protein (Fig. II-2B). Thus, although TL protein appeared to be translated normally in *grau* and *cort* embryos, TL was not a protein first translated at activation. Examining TL translation is therefore not an appropriate assay for examining the translation of proteins at

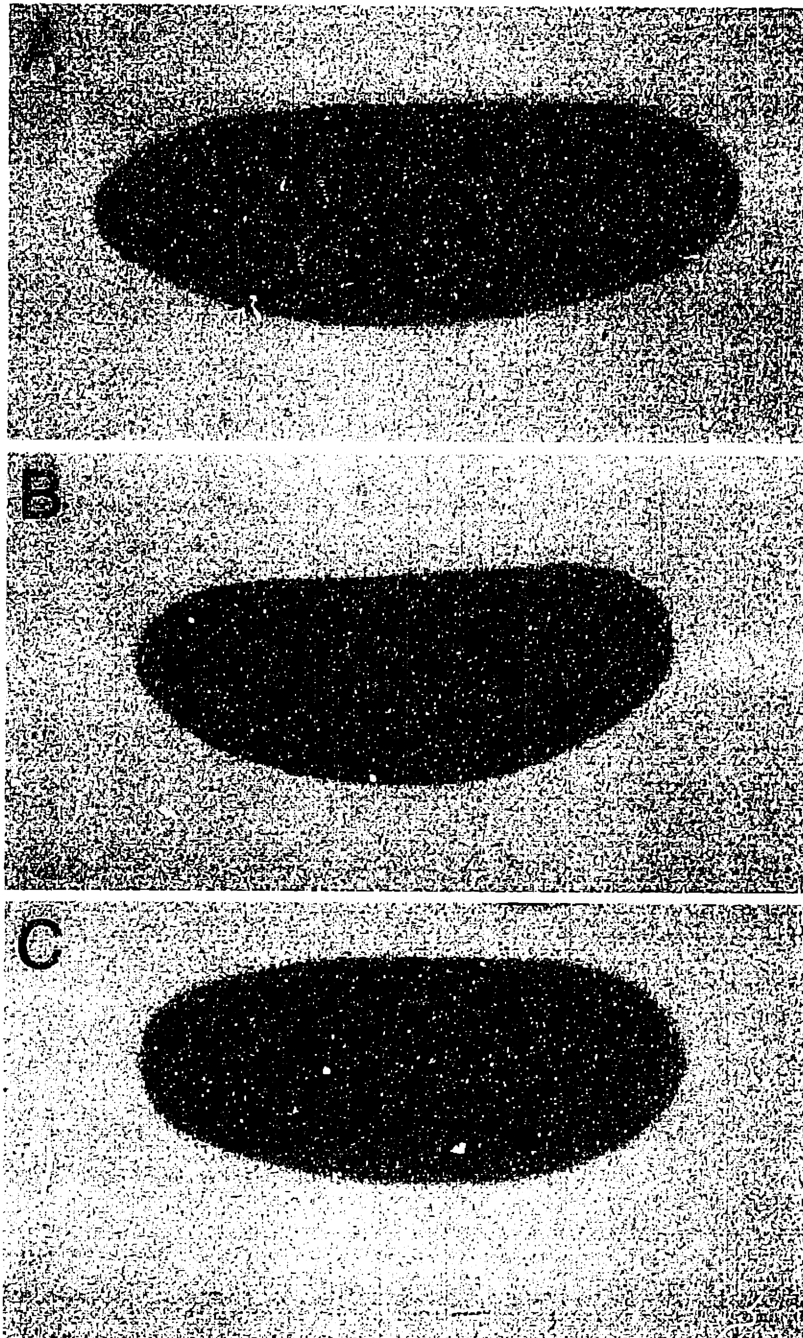


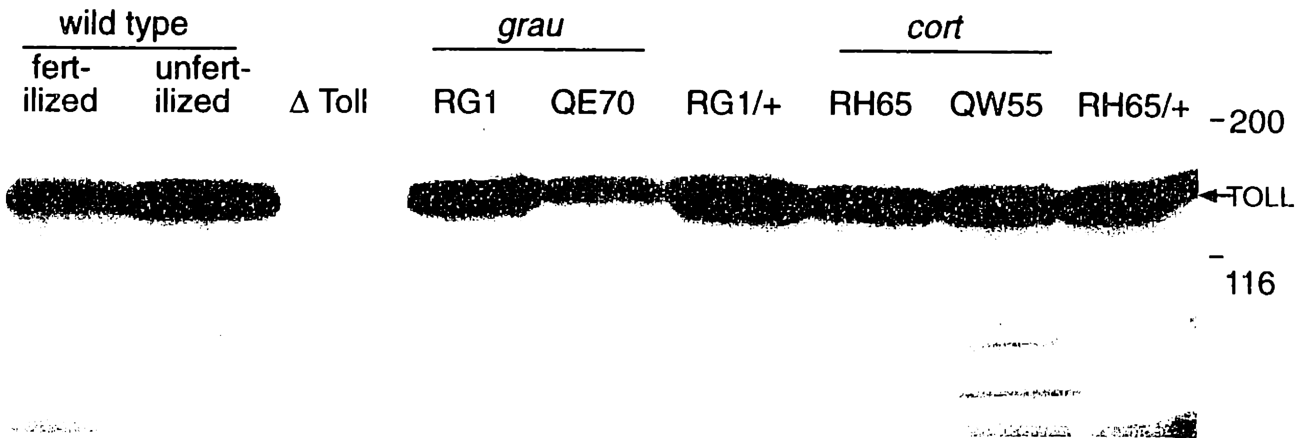
Figure II-1. BICOID (BCD) protein is not localized in *grauzone* and *cortex* embryos.

These embryos were stained with anti-BICOID antibodies from R. Lehmann. Anterior is shown to the left.

A) Embryos from *grauzone* heterozygous mother have a normal anterior gradient of BICOID protein.

B) Embryos from *cort*^{QW55} embryos have no BCD gradient.

C) Embryos from *grau*^{QE70} have no BCD gradient.



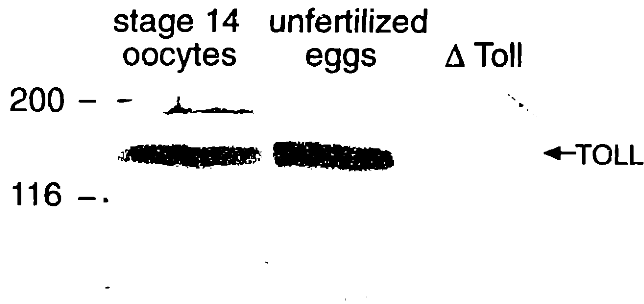


Figure II-2. TOLL is translated in *grau* and *cort* eggs.

Blots of extracts from *grauzone*, *cortex*, wild-type and *Toll* null embryos and oocytes were probed with anti-TOLL antibodies.

A) TOLL is expressed as a 135 kDa protein in eggs from mothers homozygous for $grau^{RG1}$, $grau^{QE70}$, $cort^{QW55}$, or $cort^{RH65}$, as it is in wild-type embryos.

B) TOLL is expressed in mature stage 14 oocytes before activation at approximately the same levels as after activation in unfertilized eggs.

activation.

We were unable to determine with certainty whether or not proteins are translated normally in embryos from *grau* and *cort* mothers. However, because we have established that new protein translation is not required for the completion of meiosis after the metaphase I arrest (Page and Orr-Weaver, 1997), we believe it is unlikely that *grau* and *cort* embryos fail to translate a protein essential for meiotic progression.

Methods:

For the BCD whole mount staining, embryos were fixed in formaldehyde and stained with horseradish peroxidase with the Elite Kit (Vector Laboratories) according to the method of Gavis and Lehmann (1992). Embryos were collected for 3 hours from $grau^{QE70}$, $grau^{QE70}/+$, $cort^{QW55}$, and $cort^{QW55}/+$ mothers. Anti-BCD mouse monoclonal antibodies (kindly provided by S. Strickland (Lieberfarb et al., 1996) and R. Lehmann (Driever and Nusslein-Volhard, 1988) were preabsorbed against embryos age 10-22 hours overnight at 4°C. Antibodies from R. Lehmann were used at a final concentration of 1:500 and antibodies from S. Strickland were used at a final concentration of 1:100. Embryos were incubated with primary antibodies overnight at 4°C. Biotinylated secondary antibodies (Elite Kit, Vector Laboratories) were diluted 1:500 and incubated with embryos for 2 hours at room temperature.

For TOLL immunoblots, embryos 0-2 hours old were collected from *grauzone* homozygous females of alleles *QE70*, *QQ36*, and *RG1*; *cortex* homozygous females of alleles *QW55* and *RH65*; heterozygous females *RG1/CyO* and *RH65/CyO*; *Toll* females of genotype $Df(3R)Tl^{9QRX}/Df\ (3R)\ ro^{XB3}$ (transheterozygous for *Tl* deficiencies), and wild-type *y w*, and *y w* virgins mated to sterile *XO* males (to collect unfertilized eggs). To collect stage 14 mature oocytes, *y w* females were disrupted by the blender method (described in Page and Orr-Weaver, 1997 as modified from Theurkauf and Hawley, 1992), and the resulting oocytes were examined under a dissecting microscope in IB buffer to select only mature stage 14 oocytes, based on the morphology of the dorsal appendages. Collection was completed and extracts prepared within 55 minutes of the initial disruption. *Toll* flies were a gift from C. Hashimoto.

The volume of embryos was estimated, and for every 1 µl of embryos, 5 µl of a 1:1 mixture of EB (freshly prepared; Edgar et al., 1994):4X Laemmli SB was added. Embryos were crushed in this buffer with a melted glass pipet, boiled for

10 min., and spun for 10 min. to precipitate insoluble material. The supernatant was removed to new tubes, frozen in a dry ice/ethanol bath, and stored at -80°C. Extracts were loaded on a 6% (37:1) acrylamide: bis-acrylamide gel, and after electrophoresis proteins were transferred with a semidry transfer apparatus (Hoeffer) to nitrocellulose (Schleicher and Schuell). Protein loading and transfer was checked by Ponceau S staining, which revealed that, for the blot shown in Fig. II-2A, the fertilized wild-type, *Tl*, and *QE70* lanes were all about equally under-loaded with respect to the other lanes. Blots were probed with 800 µl of a 1:40 dilution of a C-terminal anti-TOLL antibody (a gift from K. Anderson) in TBS with 3% BSA. (N-terminal antibodies from C. Hashimoto did not recognize TOLL on these blots). Secondary antibodies were alkaline-phosphatase-labeled goat anti-rabbit (Promega), diluted 1:7500 in TBS with BSA. Developing was performed with the BCIP/NBT color substrate development reagents (Promega).

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Appendix III: Activation of *grau* and *cort* Oocytes

Eggs laid by *grauzone (grau)* and *cortex (cort)* mothers arrest inappropriately in meiosis II with condensed chromosomes. One hypothesis that could explain this arrest is that they do not become properly activated as they pass through the oviduct to the uterus (Page and Orr-Weaver, 1996). A failure in egg activation would also explain why the mutants fail to reorganize their microtubule cytoskeletons and may fail to translate the BICOID protein in response to activation (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). If mutant oocytes could not be activated because the mother was defective in providing an activation signal, one prediction would be that such eggs could be activated in vitro to complete meiosis.

I activated eggs from mothers mutant for either *grau*^{QF31} or *cort*^{QW55}. For the *grau* experiment, about 37 activated eggs were recovered from 100 fattened females; for the *cort* experiment, about 200 activated eggs were recovered from about 300 fattened females. Activated eggs of both genotypes were fixed in methanol at 25, 40, and 90 minutes after activation; *cort* eggs were also fixed at 120 minutes after activation. Fixed eggs were stained to visualize DNA and tubulin. Activation protocols and staining procedures are described in (Page and Orr-Weaver, 1997).

Generally when wild-type oocytes are activated, most of them complete meiosis II within 25 minutes. Between 40 and 60 minutes after activation, many wild-type activated eggs have four meiotic products that are decondensed in an interphase-like state. At 90 minutes after activation, some nuclei have recondensed their chromosomes and arrested them in the characteristic polar body rosette typical of unfertilized eggs activated in vivo; other activated eggs have initiated mitotic cycling, which is never observed in unfertilized eggs activated in vivo.

When *grau* and *cort* oocytes were activated, meiosis II configurations were observed in about 70% of eggs 25 minutes after activation. Importantly, decondensed meiotic products were never observed at any time point. However, by 90 minutes the condensed chromosomes appeared to have changed their orientation to resemble wild-type polar bodies in about a third of the eggs. At 120 minutes, about 40% of the *cort* eggs appeared to be in the polar body configuration as judged by rosette-shaped chromosomes with a ring of tubulin pulling them

together (Fig. III-1; *grau* eggs were not examined at 120 minutes). Polar bodies are never observed in *grau* and *cort* eggs activated in vivo.

This result appears at first to be self-contradictory: meiosis II can never resolve into telophase, and the post-meiotic interphase is never observed; yet polar bodies form after two hours. My interpretation is that the meiotic arrest in *grau* and *cort* eggs, which prevents them from exiting the second meiotic division, cannot be overcome by in vitro activation. For both wild-type eggs and *grau* and *cort* mutants, in vitro activation conditions do not completely mimic in vivo conditions, and observations made more than an hour after activation are artifactual and no longer represent what happens normally.

Even if it is agreed that in vitro activation does not relieve the meiotic arrest in *grau* and *cort* eggs, the primary defect in these eggs could still be in activation. Rather than a defect in the signal sent from the mother, the mutants could be defective in receiving or transducing the signal inside the oocyte.

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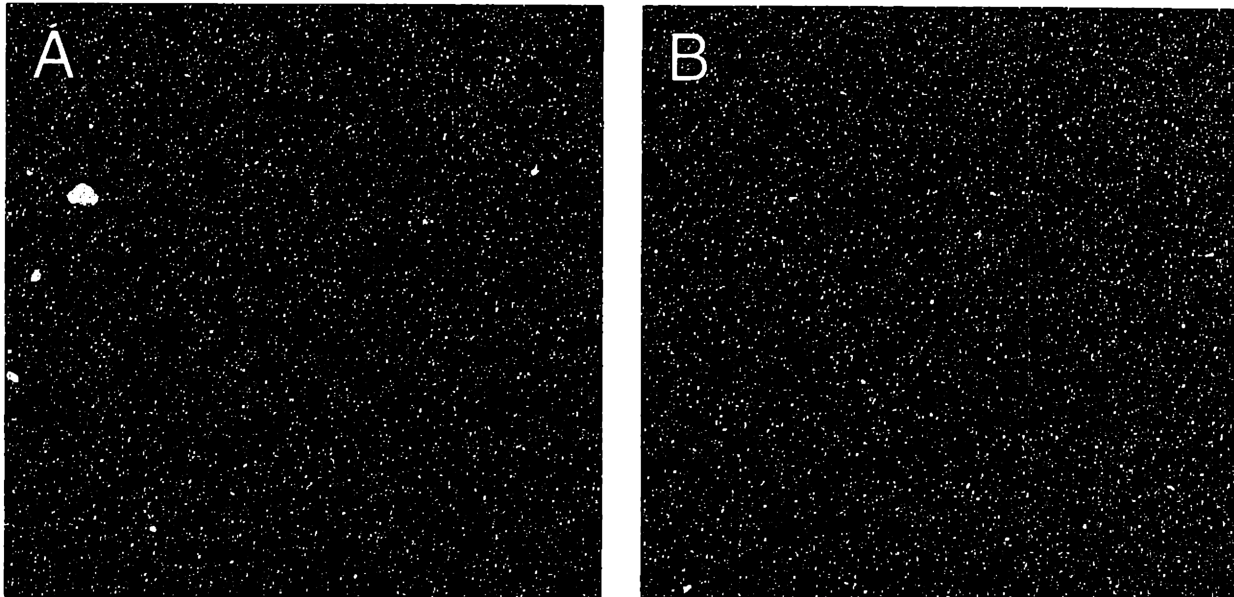

Figure III-1. Activation of oocytes from *cortex* mothers.

Eggs from females homozygous for *cortex*^{QW55} were activated in vitro. After 120 minutes, some eggs appeared to have polar body rosettes as seen here; polar bodies are never seen in mutant eggs activated in vivo. However, decondensed interphase nuclei were never observed, suggesting that the meiotic arrest in *cortex* eggs cannot be rescued by in vitro activation.

A. DNA staining with 7AAD showing the rosette chromosome arrangement.

B. Anti-tubulin staining.

Appendix IV: Protocols for In Vitro Activation of Drosophila Oocytes

Isolating and Activating Oocytes

Oocytes are isolated by first disrupting females in a blender, then filtering oocytes out of the homogenate based on size, and finally enriching for oocytes by gravity settling in a sucrose buffer. Oocytes are activated by the sequential addition of two buffers, the first containing PEG and the second a more physiological buffer demonstrated to support the growth of permeabilized embryos (Limbourg and Zalokar, 1973).

In order for oocytes to activate normally, it is critical to minimize the time between the initial disruption of females and the addition of the first activating buffer. Generally, oocytes are isolated in 15 minutes (monitored with a timer) and activated immediately. If it is desired to introduce a small molecule into the oocytes before activation, such as a radiolabeled amino acid or BrdU, this can be achieved by isolating the oocytes in 15 minutes, incubating them with the small molecule in IB for an additional 10 minutes with air bubbling through the media, then activating them. See the commentary section for trouble-shooting ideas.

Materials:

- 100-300 fattened female flies
- CO_2 source
- Blender
- IB (Isolation Buffer)
- AB (Activation Buffer)
- ZAB (Zalokar's Activation Buffer)
- 5 nylon meshes (available from Tetko, 716-683-4050)
  - 650 µm mesh, 5" x 5"
  - 250 µm mesh, 5" x 5"
  - 250 µm mesh, 3" x 3"
  - 125 µm mesh, 5" x 5"
  - 125 µm mesh, 3" x 3"
- 3 - 500 ml beakers,
- 2 - 150 ml beakers
- 1 - 30 ml beaker
- Aspirator with clean tip
- Pressurized air supply with a clean tip

The blender jar, meshes, and beakers should be rinsed with MilliQ water and dried before use to remove any possible residues.

Isolating Oocytes

1. Fill the blender with about 200 ml IB. Anesthetize the flies with CO_2, then add them all to the blender. Pulse the blender, 3 times, 2 sec each. (It is helpful to start a timer when blending first begins.)

2. Strain the homogenate through the 5" x 5" 650 µm mesh and collect the filtrate in the 500 ml beaker. (Oocytes will pass through this mesh.)

3. Return the debris on the filter to the blender by pouring about 100-150 ml fresh IB through the reverse side of the mesh into the blender jar. Pulse the blender 3 times, 2 sec each.

4. Strain the homogenate again through the 5" x 5" 650 µm mesh and pool the new filtrate with the previously collected filtrate in the 500 ml beaker.

5. Return the debris pieces on the mesh to the blender by pouring about 100-150 ml fresh IB through the reverse side of the mesh into the blender jar. In this third blending, puree the large pieces by blending continuously for about 10 sec. Strain the homogenate through the 5" x 5" 650 µm mesh and pool the new filtrate with the previously collected filtrates in the 500 ml beaker. Discard any large pieces that remain on the mesh.

6. Strain the pooled filtrate through the 5" x 5" 250 µm mesh into a second 500 ml beaker. (Oocytes will pass through this mesh.) Wash any additional oocytes through the mesh and into the beaker by pouring about 20-50 ml IB through the mesh, and repeat. Discard the debris on the filter.

7. Collect the oocytes on the 5" x 5" 125 µm mesh by pouring the filtrate through the mesh into another 500 ml beaker. (Oocytes will not pass through this mesh.) Discard the new filtrate.

8. Rinse the oocytes off the 125 µm mesh into a 150 ml beaker by pouring a little IB down the mesh.

9. Pour the oocytes through the 3" x 3" 250 µm mesh into a new 150 ml beaker. Discard the debris on the filter.

10. Collect the oocytes on the 3" x 3" 125 µm mesh by pouring the filtrate through the mesh into another 150 ml beaker. Discard the new filtrate.

11. Rinse the oocytes off the small mesh into the 50 ml beaker by pouring IB down the mesh.

12. Let them settle until most are on the bottom, (less than a minute), then aspirate off the stragglers with a vacuum aspirator, leaving about 10 ml remaining in the beaker.

13. Pour in about 40 ml IB, let the oocytes settle, and then aspirate. Repeat about 7 times, or until 15 min. after you started to blend. (The goal is to remove the non-oocytes, which settle more slowly than the oocytes. Oocytes are white and oval-shaped, and settle very quickly.)

14. Fifteen minutes after you started to blend, remove the excess IB. (If you want an unactivated control, remove them now to fixative.)

15. Optional: incubate oocytes in IB containing inhibitors or other small molecules for 10 minutes to allow entry of the molecules into the oocyte.

Activating Oocytes

1. Add about 15 ml of AB to the beaker, and note the time. This is the time of activation.

2. Remove the AB when the oocytes have settled, and replace with fresh AB. Repeatedly remove and replenish the AB until you have used 50 ml of AB. Let the oocytes sit in AB a total of five min. from when you first added it.

3. Remove the excess AB and replace it with ZAB. Wash in ZAB repeatedly by letting the oocytes settle, aspirating off the liquid, and replenishing it. Use a total of 100 ml of ZAB. After the final washes, keep the oocytes in 20-30 ml ZAB.

4. <u>Gently</u> bubble air into the oocytes in ZAB until the appropriate amount of time has passed after activation. Activated oocytes should be fixed immediately.

PREPARING ACTIVATED OOCYTES FOR CYTOLOGICAL EXAMINATION

Materials

50 ml beaker for each time point
50% bleach solution (50% Chlorox bleach / 50% 0.7% NaCl, 0.05% TX-100) freshly prepared
small nylon, nytex, or wire filter for collecting embryos and/or oocytes
0.7% NaCl / 0.05% TX-100
Cacodylate buffer / formaldehyde fixative
paintbrush
1 dram vial for each time point

PBST (PBS with 0.3% Triton-X 100)
PBST + 1% BSA (freshly prepared or thawed)
dissecting microscope
microscope slides with frosted ends
25 ml beaker for each time point
microfuge tube for each time point

Dechorionating and Selecting Activated Eggs

1. At the appropriate time after activation, remove an aliquot of activated eggs and buffer to a 50 ml beaker. Add about 25 ml 50% bleach solution. Swirl for 3 minutes. Pour the bleach-oocyte mixture onto a small filter to remove the bleach, and gently wash the oocytes on the filter with 0.7% NaCl / 0.05% TX-100.

Fixing Activated Eggs in Formaldehyde

1. Add 2 ml cacodylate buffer / formaldehyde to a 1 dram vial. Remove the oocytes from the filter with a paintbrush, and add them to the fixative in the vial. Rock gently for 10 minutes.

2. Remove the fixative carefully with a Pasteur pipet and discard. Wash the oocytes three times in PBST. (If desired, toocytes may now be transfered to PBS and stored overnight at 4°C.)

Devitellinizing Formaldehyde Fixed Eggs

Vitelline membranes are removed by friction as eggs are lightly squashed between the frosted sides of two glass slides. The slides are rubbed back and forth, with slight changes of direction, to break open the vitelline membrane. Progress is monitored under a dissecting microscope. This skill is learned over time: too little rolling and the membranes remain attached; too much rolling and the eggs are pulverized.

1. Gently rub together the frosted sides of two glass slides for about 10 seconds to soften the frosted surfaces. Rinse off the glass dust with water and dry the slides. One of these will be the bottom slide and one the top slide.

2. Under a dissecting microscope, set up 3 microscope slides for rolling eggs: the two rubbed slides and another slide as a base. Place one rubbed slide, frosted side up, under the scope. Place another (non-rubbed) slide nearby to provide a stable base, and label it with the letter "B" to avoid confusion later. Put a drop of PBST + BSA onto the frosted surface, and spread the liquid by gently rubbing top slide's frosted surface against the bottom slides' frosted surface. (The BSA acts to prevent eggs from sticking to the slides.)

3. Draw some PBST + BSA into a Pasteur pipette to lubricate the pipette with BSA, and expel it. Draw some fixed eggs into the pipet, and let them settle at the bottom of the pipette. Add the eggs in a drop of PBST to the frosted surface of the bottom slide. Gently place the top slide, frosted side down, onto the bottom slide. If there is too much liquid with the eggs, it will leak out the sides; in that case remove the top slide and try to absorb some liquid with a Kimwipe. If there is not enough liquid, the slides will tightly adhere to each other; in that case, carefully remove the top slide and add a bit more PBST.

4. Rub the slides back and forth, gently rolling the eggs. The eggs will align themselves perpendicular to the direction of motion if there is appropriate pressure. To keep the angle of the top and bottom slides the same, rest the back (unfrosted) end of the top slide on the "B" base slide. If there is much liquid between the two slides, the eggs will not align; remove the top slide and try to remove some of the liquid with a Kimwipe.

5. After the eggs have aligned themselves in one direction, gently begin to change the direction of the rubbing motion. If the original motion defines a line at 0°, change to rubbing along a line at a 20° angle, then a 40° angle, and so on, until you return to the original angle.

6. Monitor the progress under the dissecting microscope. To remove the top slide, raise the slide at an angle, keeping one corner down, and simultaneously bring that corner into the center of the bottom slide. This will concentrate the oocytes in the middle of the frosted surface.

7. When about 75% of the eggs have lost their vitelline membranes (visible as shiny, waxy surfaces), stop rolling. Lubricate a 25 ml beaker with PBST + BSA to prevent the oocytes from sticking, and rinse the oocytes off the slides into the beaker.

8. Lubricate a Pasteur pipette with PBST + BSA, and vigorously draw up and release eggs repeatedly to dislodge any vitelline membranes that are still adhering to the eggs.

9. Wash the eggs in PBST several times, letting eggs settle and aspirating off the more slowly migrating vitteline membranes, visible as cloudy particles.

10. If a particularly clean preparation is desired, the entire procedure can be repeated on the same sample. Repetition is a more successful strategy than simply rolling for longer the first time.

11. Collect the embryos in a microfuge tube.

12. If staining with antibodies is desired, permeabilize the eggs with a 1-2 hour incubation in 1% TX-100 in PBS.

13. Wash 2X in PBST. Eggs are now ready for staining.

Devitellinizing and Fixing Activated Eggs in Methanol (Alternate Protocol)

Materials

Scintillation vial for each time point
methanol
heptane
90% methanol/10% PBS
75% methanol/25% PBS
50% methanol/50% PBS
25% methanol/75% PBS
PBST

1. After dechorionating eggs and washing eggs, wash them from the filter into a 20 ml scintillation vial with heptane. Add or remove heptane so that there is 5 ml in the vial.

2. Add all at once 5 ml of methanol, quickly place the cap on the vial, and immediately shake the vial vigorously. After about a minute of shaking, let the methanol and heptane layers separate. Eggs that sink to the bottom have been devitellinized.

3. Discard the heptane, eggs at the interface, and most of the methanol layer. Replace with 10 ml of fresh methanol. Incubate at room temperature 3 hours, or overnight at 4°.

4. Rehydrate eggs through a methanol/PBS series: remove methanol and add 90% methanol/10% PBS, let sit for 1 minute. Remove this, add 75%/25%, let sit for 1 min. Remove, add 50%/50%, let sit for 1 min. Remove, add 25%/75%, let sit for 1 min. Remove, wash 2X in PBST, and let sit in PBST. Eggs are now ready for antibody staining.

Reagents and Solutions

Make up solutions in glassware that has been rinsed in MilliQ water. AB, ZAB, and IB may be made up in large quantities and frozen as concentrated or 1X stocks. IB, AB, and ZAB should be stored at 4°, and used at room temperature within a day of preparation or thawing. Use only the purest MilliQ water available for IB, AB, and ZAB.

IB (Isolation Buffer, adapted from (Theurkauf and Hawley, 1992)
55 mM NaOAC
40 mM KOAc
110 mM sucrose
1.2 mM $MgCl_2$
1 mM $CaCl_2$
100 mM HEPES
pH to 7.4 with NaOH

AB (Activation Buffer, adapted from (Mahowald et al., 1983)
3.3 mM NaH2PO4
16.6 mM KH2PO4
10 mM NaCl
5% PEG 8000
2 mM CaCl2
pH to 6.4 with 1:5 NaOH:KOH.

ZAB (Zalokar's Activation Buffer, adapted from (Limbourg and Zalokar, 1973)
9 mM MgCl2
10 mM MgSO4
2.9 mM NaH2PO4
0.22 mM NaOAc
5 mM glucose
34 mM glutamic acid
33 mM glycine
2 mM malic acid
67 mM CaCl2
pH to 6.8 with 1:1 NaOH:KOH

Cacodylate Buffer/formaldehyde (Theurkauf and Hawley, 1992)
2X Cacodylate Buffer
200 mM cacodylic acid
200 mM sucrose
80 mM KOAc
20 mM NaOAc
20 mM EGTA
pH to 7.2 with KOH and filter sterilize
Just before using, mix equal volumes of 2X Cacodylate Buffer and 16% EM-grade formaldehyde (Ted Pella).

10X PBS
1.3 M NaCl
70 mM Na_2HPO_4
35 mM NaH_2PO_4
pH to 7.0 and autoclave
Dilute to 1X before using.

COMMENTARY

There are a number of variables to consider when activating oocytes. The first question is what stage of meiosis is wanted for observation because that will determine how long after activation the eggs are fixed. Eggs do not progress through meiosis in tight synchrony, and some appear never to exit meiosis I, but nonetheless how much time has elapsed between activation and fixation will determine what stages of meiosis are prevalent in the collection. Some indication of the relationship between timing and stage of meiosis can be gained from Table 3-1; for the earlier stages I have generally observed that meiosis I can be visualized within 5-10 minutes and meiosis II in 15-25 minutes after activation. The timing of the meiotic divisions in vitro varies somewhat, so it may be necessary to determine when to fix the eggs in preliminary experiments.

Fixation methods

The stages of meiosis desired and the time required to achieve those stages will then determine what fixation method to use. Generally, the methanol/heptane method of fixing, devitellinizing, and permeabilizing is substantially simpler than the formaldehyde method. It is also reliable for allowing antibody penetration into the eggs, and spindle staining is much easier to visualize in methanol-fixed eggs. However, the disadvantage of the methanol/heptane method is that newly activated eggs, under about 25 minutes, will not be devitellinized and so cannot be observed. After about 25 minutes, some eggs can be devitellinized in methanol/heptane, and after 40 minutes most can. Methanol fixation may be reasonable for viewing meiosis II and the four meiotic products, but not earlier stages.

It may be desirable to vary the length of formaldehyde fixation. Too much fixation and antibodies cannot enter; too little fixation and structures are not preserved. The ability of formaldehyde to fix eggs through the vitelline membrane may vary with the length of time the eggs have been activated, so this may need to be optimized. Also consider how long it takes to remove the formaldehyde from the vial when considering the length of fixation time.

This protocol includes a selection for activated eggs in 50% bleach. Mature stage 14 oocytes are destroyed by exposure to 50% bleach; upon activation, they become impermeable to bleach. Incubation in bleach also serves to remove the chorions of activated eggs. If a selection is not desired, it is possible to fix activated eggs directly in 100% methanol without dechorionating or devitellinizing. Without devitellinizing, however, antibodies will not be able to penetrate the egg. Although not extensively tested, it is also possible to fix directly in formaldehyde without bleach selection. Both the chorions and the underlying vitelline membranes can then be removed with rolling between frosted slides (see protocol). If followed by permeabilization in 1% Triton X-100, antibody penetration should be possible. In addition to lacking a selection for activated eggs, another drawback of this method is that follicle cells associated with the chorion can obscure the oocyte nucleus (see the below).

Limitations of the in vitro system

Cytologically, it appears that many eggs proceed through meiosis normally, with timing comparable to in vivo activated eggs. However, since a few abnormal events are always observed, it is important to observe many eggs in a particular stage of meiosis before drawing conclusions. Also, after the recondensation of the meiotic products, artifacts appear. Great variability is observed: some nuclei form polar bodies, some replicate without dividing, and some enter the cell cycle. Because this variability is not observed in laid eggs, it is important not to use the in vitro system to examine events after the recondensation of the meiotic products. Such events can be examined in short collections of laid eggs without substantial difficulty.

Trouble-shooting

If in vitro activation does not work the first time, consider the following sources of error and artifact. First, were many oocytes isolated from the females? If several hundred oocytes were not isolated before activation, then the number of females should be increased, they should be fattened for more or less time, or they should be younger. It is also possible that oocytes were inadvertently aspirated away during the gravity settling steps.

Were oocytes or eggs lost by sticking to the glassware? Oocytes especially can be very sticky. If each mesh and piece of glassware is wetted with IB just before contacting the oocytes, they will stick considerably less. Also oocytes can be washed off of glassware with IB squirted through a pipette, and beakers can be continually swirled so that oocytes cannot settle.

Were oocytes able to be activated, i.e., did they survive bleach treatment? If not, perhaps it took longer than 15 minutes to isolate them. Longer isolation times may adversely affect activation frequency. Also consider remaking the IB, AB, and ZAB solutions. It is essential that the glassware, cylinders, etc., are completely clean and free of soap and other residues when the solutions are being made. Consider rinsing each item several times with MilliQ water before using. Also, the pH and ionic strength of each solution may be important. When adjusting the pH of these solutions, make sure not to overshoot the mark and then compensate, as this changes the final composition of the buffer.

If the eggs clump together when their vitelline membranes are still present, the addition of more Triton or BSA may help to disrupt the interaction between the waxy membranes. If they clump together without their vitelline membranes, and cannot be separated easily, then it is likely that there is substantial microbial contamination (see below).

Identifying the meiotic stages

The most difficult aspect of in vitro activation can be recognizing the meiotic stages. Meiosis occurs in the dorsal anterior quadrant of the oocyte. Although this quadrant can be identified by its characteristic shape in laid eggs, the rolling procedure can disrupt the morphology so that it is difficult to locate the quadrant. Some other landmarks may help to orient the observer. First, many DNA stains appear to stain the posterior pole faintly, perhaps because of a

high concentration of mitochondria. Second, the micropyle is sometimes visible as a small blip on the anterior end of the egg. Third, the dorsal side can sometimes be identified by its flat V-shaped operculum, visible when the dorsal side is facing up. Meiosis I figures lie at the point of the operculum; meiosis II spindles lie end-to-end in the same region. The four meiotic products are arranged colinearly during telophase II. The three outermost meiotic products cluster together, appearing to overlap, during the post-meiotic interphase.

It can be very difficult to observe the four decondensed meiotic products after meiosis because the DNA is so diffuse that it does not stain very brightly. This is even more true if the eggs have been treated with cycloheximide so that the nuclei do not recondense. A more intense DNA stain may help, such as DAPI or propidium iodide. Also, tubulin staining can help enormously in identifying the decondensed meiotic products because, although no spindles are visible at this time, the meiotic products will be visible as clearings in the cytoplasmic staining of depolymerized tubulin.

If activated eggs were recovered, but it was difficult to determine the meiotic stage, several factors might be contributing to the difficulty. If it was difficult to identify the nucleus because of many possible candidates, contaminating materials such as bacteria, follicle cells, or dust may be the culprit. It is essential that no solutions have microbial contamination. This can be avoided by using freshly made solutions or by freezing solutions after preparation and then thawing just before use. Once eggs are fixed, preservatives such as sodium azide and thimerosol can be added indiscriminately to all solutions to retard microbial growth. Solutions with BSA are especially prone to microbial growth, and Triton-containing solutions are also potential sources of contaminants. Another form of contamination can be the follicle cell nuclei surrounding the chorion. If the chorions were not removed with bleach, and instead are removed by rolling, the follicle cells can become displaced all across the surface of the egg. These can completely obscure the oocyte nucleus/nuclei. For eggs with intact chorions, it might be necessary to roll and wash them twice to completely remove all the follicle cell nuclei. Also, tubulin staining can help enormously in recognizing meiosis I and II.

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Thesis Song of J. Alfred Proofreader[†]

Giammai di questo fondo
Non torno vivo alcun

Let us go then, you and I,
When my research is spread out before my eyes
Like a patient etherized upon a table;
Let us go, through half-deserted stacks,
The anxiety attacks
Of restless nights in all-night thesis hell,
In library stacks where the hopeless dwell:
Stacks that follow like a tedious argument
Of insidious intent
To lead you to an overwhelming question . . .
Oh, do not ask, "What is this for?"
Let us go face the Inquisitor.

In the room professors come and go
Talking of things we do not know.

And indeed there will be time,
To do the work my thesis still requires,
A last minute treat.
There will be time, there will be time
To write a tome to give the readers that you meet;
There will be time to murder and create,
And time for all the works and days of hands
That lift and drop a question on your plate;
Time for you and time for me,
And time yet for a hundred indecisions,
And for a hundred visions and revisions,
Before the meeting of the committee.

In the room professors come and go
Talking of things we do not know.

[†] With apologies to T. S. Eliot

And indeed there will be time
To wonder, "Do I dare?" and "Do I dare?"
Time to turn back and escape the academic snare,
With ordinary dress at that Commencement fare --
[They will say, "Why is she still here?"]
My work begun in a year beyond their ken,
My class banner, dating back to who knows when --
[They will say, "Has she no plans yet for next year?"]
Do I dare
Disturb the thesis curse?
In a minute there is time
For decisions and revisions which a minute will reverse.

For I have known them all already, known them all:
Have known the evenings, mornings, afternoons,
I have measured out my life with coffee spoons;
I know computers dying with a dying fall
Beneath the frolic from a farther room.
        So how should I presume?
And I have known the examiners already, known them all --
Their eyes that fix you in a formulated phrase,
And when I am formulated, sprawling on a pin,
When I am pinned and wriggling on the wall,
Then how should I begin
To spit out the butt-ends of my research waste?
        And how should I presume?

And the afternoon, the evening, sleeps so peacefully!
Smoothed by long fingers,
Asleep . . . tired . . . or it malingers,
Stretched on the floor, here beside my books and me.
Should I, after take-out food surprises,
Have the strength to force my research to its crisis?
But though I have wept and fasted, wept and prayed,
Though I have seen my head brought in upon a platter,
I am no scholar -- and here's no great matter;
I have seen the moment of my greatness flicker,
And I have seen the eternal Reader hold my text, and snicker,
And in short, I was afraid.

And would it have been worth it, after all,
After the plates, the carry-out cartons, the tea,
Among the coffee cups, among some talk of a law degree,
Would it have been worthwhile,
To have bitten off the matter with a smile,
To have squeezed the universe into a ball
To roll it toward some overwhelming question,
To say: "I am a Graduate Student, come from the dead,
Come back to tell you all, I shall tell you all" --
If one, in a meeting that I dread,
        Should say: "That is not it at all.
        That is not what you said, at all."

No! I am not Tenured Professor, nor was meant to be;
Am an attendant aid, one that will do,
To swell a paper, start a chapter or two,
Advise the prof; no doubt, an easy tool,
Deferential, glad to be of use,
Politic, cautious, and meticulous,
Full of high sentence, but a bit obtuse;
At times, indeed, almost ridiculous --
Almost, at times, the fool.

I grow old . . . I grow old . . .
I shall find my diploma still on hold.

Shall I part my chapters so? Do I dare to go to sleep?
I shall rehearse my arguments and attempt the final leap.
I have heard the muses singing, each to each.

I do not think they will sing to me.

We have lingered too long in the academic sea,
Not sinking, not swimming, not anchored down,
Till readers' comments shake us, and we drown.