Activation of the Meiotic Divisions in Drosophila Oocytes

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Key meiotic events in many organisms are controlled at the translational level. In this study, we examine the role of translational regulation in the meiotic cell cycle of Drosophila. 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 female 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 oocytes, synthesis of new proteins is required for release from the prophase I arrest, and for entry into meiosis II (Wasserman and Masui, 1975; Gerhart et al., 1984). In clam and starfish oocytes, 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 oocytes of 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; Theurkauf et al., 1992; Page and Orr-Weaver, 1996). 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 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 min (Riparbelli and Callani, 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 dissection or rapid collection of a few or single laid eggs (Huetten, 1924; Sonnenblick, 1950; Dävring and Sunner, 1973; Riparbelli and Callani, 1996). Since most mutants produce eggs with reduced frequency, only healthy wild-type stocks are amenable to such studies, and so there are few cytological descriptions of meiotic mutants beyond the metaphase I arrest. Because of these constraints, 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 quantities of oocytes (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 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 MgCl₂, 1 mM CaCl₂, 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- to 15-sec 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 min from the time of the first blender pulse, and resulted in populations highly enriched for unactivated stage 13 and 14 oocytes. An additional 10-min 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 min (3.3 mM NaH₂PO₄, 16.6 mM KH₂PO₄, 10 mM NaCl, 50 mM KC1, 5% PEG 8000, 2 mM CaCl₂, 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 Limbour and Zalokar to support growth of embryos with permeabilized vitelline membranes. (Limbour and Zalokar, 1973; ZAB: 9 mM MgCl₂, 10 mM MgSO₄, 2.9 mM NaH₂PO₄, 0.22 mM NaOAc, 5 mM glucose, 27 mM glutaric acid, 33 mM glycine, 2 mM malic acid, 7 mM CaCl₂, 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 three min incubation in 50% bleach (data not shown), indicating that the vitelline membranes had not become permeable to experimental manipulation. Second, DNA staining of oocytes isolated and incubated in IB for various times up to 60 min revealed that such oocytes remained arrested at metaphase I (Table 2, Fig. 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 impair 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 min as above were then incubated for exactly 10 min in fresh IB containing 0.1–1 mCi/ml of [35S]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
FIG. 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) 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 chromosome 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. (A) Adapted from Theurkauf and Hawley (1992); (B–F) adapted from Huettner (1924).

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 min and 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 min were often lost at the devitellinization step. Therefore, to view the early stages of meiosis, we dechorionated as above and then fixed for 10 min 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 hr before antibody labeling.

For spindle staining, eggs were incubated with a mouse monoclonal antibody against α-tubulin (Amersham) at a concentration of 375 ng/ml in PBST (130 mM NaCl, 70 mM Na2HPO4, 35 mM NaH2PO4, 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 5 μg/ml 7-AAD (Molecular Probes) for 30 min, or by staining with OliGreen (Molecular Probes) diluted 1:5000 in PBS with 0.1% Triton and 20 μg/ml of RNase A for 30 min.

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 5× and 40× 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 40× 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 [35S]-methionine, activating, dechorionating, 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 coverslip 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 appendage tube of methanol, which dissolved the clearing solution. Eggs (5–20) were collected for each sample, and these were rehydrated in PBS and 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:Laemmli sample buffer was added [EB, described by Edgar et al. (1994): 10 mM Tris, pH 7.5, 80 mM Na2/glycophosphate, pH 7.5, 20 mM EGTA, 15 mM MgCl2, 2 mM Na3VO4, 1 mM benzamidine, 1 mM sodium metabisulfite, 0.2 mM PMSF] in a volume of 0.5–1 μl per egg. Lysates were boiled for 10 min, stored at −20°C, and boiled for 10 min before loading. Samples were run on 14 or 16% (acrylamide:bis) 0.75-mm gels, which were stained with Coomassie blue. Incorporation of counts was quantitated for each sample, and this was compared to the background incorporation in oocytes that had been devitellinized with NIH Image software. By calibrating the program with known amounts of proteins, we ensured we were working within a range of linear response.
FIG. 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 Fig. 1. DNA is represented in red and tubulin in green. (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
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 (Huettner, 1924; Sonnenblick, 1950; Davring and Sunner, 1973; Riparbelli and Callaini, 1996). The mature oocyte arrests in metaphase of meiosis I, waiting to be activated during passage through the oviduct (Fig. 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 Fig. 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. 1D). In an unfertilized egg, the interphase nuclei migrate together, recondense their chromatin (Fig. 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. 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 nucleus.

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 visible method (Theurkauf, 1994) that allowed us to harvest many hundreds of oocytes to use as our starting material (Fig. 2A). 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. 2). We fixed activated eggs in either methanol or formaldehyde and stained them with anti-tubulin antibodies and a DNA stain to observe the stages of meiosis. Eggs undergoing meiotic divisions were clearly identified by the presence of meiosis I or two meiosis II spindles (Figs. 2B–2D). 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 (Puro and Nokkala, 1977; Hatsumi and Endow, 1992). With our method, eggs continued into meiosis II (Fig. 2D), the postmeiotic interphase (Fig. 2E), and the recondensation of chromatin to form the rosette structures (Figs. 2F and 2G). 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. 2A). In the chromatin mass at the plate, we observed no chromosome arms, individual chromosomes, or visible structures 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 condensation (Figs. 2B and 3). These morphological changes occurred before poleward movement was observed. We believe that 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 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 min, fixed directly in methanol, stained with a DNA stain, and examined for meiotic stage. Examining over 100 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 min, 22% of the in vitro activated eggs were in meiosis II, and another 71% had finished both meiotic divisions. After 40 min, over 90% of the eggs had completed the meiotic divisions (Table 1 and Fig. 4A). Studies on the timing of the meiotic divisions in laid eggs have reported that 20 min 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 normal postmeiotic interphase (Fig. 2E), and in many of them the chromatin of the meiotic products recondensed and fused to form rosette structures (Figs. 2F and 2G). Instead of arresting with rosette structures as would an unfertilized laid egg, however, the nuclei of eggs activated in vitro often replicated and divided 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 min after the completion of meiosis, whereas in these experimental eggs, rosette structures were much slower to form (Table 1 and Fig. 4A). This timing and behavior suggests that our in vitro activation method is useful for studying events through the postmeiotic 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

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<th>TABLE 1</th>
<th>The Stages of Meiosis Observed at Different Times after In Vitro Activation</th>
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*At the appropriate times after activation, activated eggs were stained with bleached devitellinized fixed in methanol, and immunostained with anti-tubulin and a DNA stain.*
are recruited into polysomes, suggesting that the rate of new protein synthesis is significantly enhanced (Mahowald et al., 1983). Additionally, it is known that some proteins, such as BICOID and STRING, 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 (Gerhart et al., 1984; Kanki and Donoghue, 1991; Clarke and Masui, 1983; Fulka Jr. et al., 1994; Hunt et al., 1992; Galas et al., 1993; 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 mutants grauzone and cortex arrest at meiosis II and also display defects in translation (Page and Orr-Weaver, 1996; Lieberfarb et al., 1996). We tested this hypothesis by activating eggs in the presence of the translation 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-min 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. 5A; Riparbelli and Callaini, 1996). We also observed many eggs with four condensed colinear 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 postmeiotic interphase (Fig. 5B).

The timing of the meiotic divisions was similar between cycloheximide and untreated activated eggs: after 25 min, 39% of the cycloheximide-treated eggs were in the meiotic divisions, compared to about 29% of control eggs (Table 1 and Fig. 4); and for both cycloheximide- and drug-treated eggs, about half of them were in the postmeiotic interphase after 25 min. Interestingly, decondensation progressed to form abnormally large nuclei that were visible as large areas of clearing in the cytoplasm (Fig. 5C). After 90 min or so, the DNA recondensed within these large nuclei, but the large cleared areas persisted, indicating the nuclear envelope did not break down (Fig. 5D; Table 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 postmeiotic interphase.

In order to ensure that cycloheximide had entered the oocyte and blocked protein synthesis, we metabolically labeled oocytes with [35S]methionine and then examined incorporation of the label into total protein extracts. Eggs were isolated in buffer with or without cycloheximide, labeled, activated, and 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 chosen 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. 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 fivefold did not decrease the total protein synthesis (data not shown), we considered the possibility that the remaining cycloheximide-
FIG. 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 min 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. (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. Scale bars are approximately 20 μm.

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 chosen to make protein extracts, we found that the level of incorporation of [35S]methionine was reduced to about 1% of control extracts in multiple experiments (Fig. 6). The most credible explanation is that no new proteins are required for the completion of meiosis after the metaphase arrest in Drosophila females. A protein not 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). Al-
though 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 proteins 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 backward 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 medium for 30 or 60 min, 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 polar-to-pole axis of the spindle (Theurkauf and Hawley, 1992; Fig. 2A), probably because of the tension from the spindle pulling on attached homologues; anaphase I and meiosis II can also be assessed from nuclear morphology. After 60 min of incubation in control medium, oocytes did not proceed into anaphase I and remained arrested at metaphase I (Fig. 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 medium had elongated nuclei typical of metaphase I (Table 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 morphologies in oocytes incubated in cycloheximide with roughly the same frequency (Fig. 7B; Table 2), demonstrating that new proteins, such as cyclins, are not required for 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. 7C; Table 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 visible arms (Huetter, 1924; Riparbelli and Callaini, 1996), it has been
unclear if the cytological differences observed between amorphous and individuated chromosomes were caused by difference 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. Hawley and colleagues have 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, 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 (McKim et al., 1993; Jang et al., 1995). This lack of chromosome structure implies that the normal pathway for the resumption of meiosis is not controlled by only the chromosomes. 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 postmeiotic interphase are synthesized before the arrest. Proteins re-

### TABLE 2
Cycloheximide Has No Visible Effect on Metaphase-Arrested Nuclei

<table>
<thead>
<tr>
<th></th>
<th>Round nucleus (prophase/no spindle) (%)</th>
<th>Elongated nucleus (metaphase-arrested) (%)</th>
<th>Two nuclei (anaphase or meiosis II) (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated oocytes</td>
<td>33</td>
<td>67</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>Cycloheximide-treated oocytes</td>
<td>28</td>
<td>72</td>
<td>0</td>
<td>71</td>
</tr>
<tr>
<td>Colchicine-treated oocytes</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

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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 (Gerhart et al., 1984; Kanki and Donoghue, 1991; Clarke and Masui, 1983; Fulka Jr. et al., 1994; Hunt et al., 1992; Galas et al., 1993; 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 drop 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 (Kanki and Donoghue, 1991; Furuno et al., 1994). 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 (Picard et al., 1985; Hunt et al., 1992). 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 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 posttranslational regulation.

We also tested whether the maintenance of the meiotic arrest at metaphase I required a continuing supply of new proteins. Since inhibiting protein synthesis 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, and the nuclei change to an interphase appearance (NeÂant and Guerrier, 1988; Clarke and Masui, 1983; Fulka Jr. et al., 1994; Moos et al., 1996). For Patella oocytes, two of the proteins required to maintain the arrest are cyclins A and B, since ablating these messages during metaphase I leads to the same decondensed nuclear phenotype as a 40-min treatment with cycloheximide (van Loon et al., 1991). Drosophila metaphase-I-arrested nuclei, in contrast, maintain a metaphase-I 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 (Zalokar and Erk, 1976; Edgar and Schubiger, 1986). 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. Proteins 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.

ACKNOWLEDGMENTS

We are grateful to Patrick S. McCaw and Sharon Bickel for helpful suggestions and guidance; this study would not have been possible without them. For thoughtful readings of the manuscript, we thank Andrew Murray, Jennifer Mach, Maricarmen Planas-Silva, and Doug Fenger. This work was supported by an NSF predoctoral fellowship to A.W.P. and NIH Grant GM 39341 to T.O.-W.

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Received for publication December 9, 1996
Accepted January 13, 1997