

Dose-dependent Relationship between Oocyte Cytoplasmic Volume and Transformation of Sperm Nuclei to Metaphase Chromosomes

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Abstract. We have studied the chromosome condensation activity of mouse oocytes that have been inseminated during meiotic maturation. These oocytes remain unactivated, and in those penetrated by up to three or four sperm, each sperm nucleus is transformed, without prior development of a pronucleus, into metaphase chromosomes. However, those penetrated by more than four sperm never transform any of the nuclei into metaphase chromosomes (Clarke, H. J., and Y. Masui, 1986, *J. Cell Biol.* 102:1039–1046). We report here that, when the cytoplasmic volume of oocytes was doubled or tripled by cell fusion, up to five or eight sperm nuclei, respectively, could be transformed into metaphase chromosomes. Conversely, when the cytoplasmic volume was reduced by bisection of oocytes after the germinal vesicle (GV) had broken down, no more than two sperm could be transformed into metaphase chromosomes. Thus, the capacity of the oocyte cytoplasm to transform sperm nuclei to metaphase chromosomes was proportional to its volume. The contribution of the nucleoplasm of the GV and the cytoplasm outside the GV to the chromosome

condensation activity was investigated by bisecting oocytes that contained a GV and then inseminating the nucleate and anucleate fragments. The anucleate fragments never induced sperm chromosome formation, indicating that GV nucleoplasm is required for this activity. In the nucleate fragments, the capacity to induce sperm chromosome formation was reduced as compared with whole oocytes, in spite of the fact that the fragments contained the entire GV nucleoplasm. This implies that non-GV cytoplasmic material also was required for chromosome condensation activity. When inseminated oocytes were incubated in the presence of puromycin, the sperm nuclei were transformed into interphase-like nuclei, but no metaphase chromosomes developed. However, when protein synthesis resumed, the interphase nuclei were transformed to metaphase chromosomes. These results suggest that the transformation of sperm nuclei to metaphase chromosomes in the cytoplasm of mouse oocytes requires both the nucleoplasm of the GV and non-GV cytoplasmic substances, including proteins synthesized during maturation.

DURING metaphase of meiosis or mitosis, the chromosomes become condensed under the influence of factors that appear in the cytoplasm. This cytoplasmic control of chromosome behavior has been investigated using oocytes that are undergoing meiotic maturation (reviewed in references 12 and 32). During maturation, oocytes previously arrested at prophase of meiosis advance to metaphase. In the mouse, maturing oocytes undergo the first meiotic division, progress to metaphase of the second meiotic division without an intervening interphase (13), and then become arrested at this point until fertilization activates them (reviewed in reference 29). Thus, the oocyte chromosomes, which become condensed when maturation begins, remain condensed until after fertilization. When nuclei from other cells are introduced into a maturing oocyte, by microinjection or cell fusion, their chromosomes become condensed to a metaphase state within a few hours (1, 2, 3, 19, 34, 50). Hundreds of brain nuclei can undergo this transition to metaphase within a single frog oocyte (31, 51), and even in the much smaller oocyte

of the mouse at least 15 thymocyte nuclei will condense to metaphase (10). Thus, the cytoplasm of the maturing oocyte contains a powerful activity that can stimulate the transition of many nuclei to a metaphase condition.

Recently, we examined the effect of mouse oocyte cytoplasm on sperm chromatin, which had been introduced into the cytoplasm by insemination of zona-free oocytes at prometaphase I or metaphase I of maturation (9). Under these conditions, the oocytes remained unactivated and continued maturation to metaphase II. Meanwhile, the nuclei of the incorporated sperm, without prior development of a pronucleus, were transformed directly into metaphase chromosomes. However, the number of sperm which could be transformed into metaphase chromosomes within one oocyte was limited to a maximum of three or four. When an oocyte was penetrated by more than four sperm, none of the nuclei was transformed to metaphase chromosomes. Instead, each formed a mass of condensed chromatin within the oocyte cytoplasm. These results suggested that there was a limit to

the capacity of the cytoplasm of the mouse oocyte to transform sperm nuclei to metaphase chromosomes.

A limit of the cytoplasmic activity of a single oocyte to transform sperm nuclei to pronuclei has previously been observed in mammalian oocytes (20, 49). It also has been shown that male pronuclear formation requires nucleoplasmic factors released from the germinal vesicle (GV)¹ into the cytoplasm of the oocyte (4, 46). However, no similar investigations have been carried out to examine the effects of the oocyte cytoplasm and of GV material on the transformation of the sperm nucleus into metaphase chromosomes in mammalian species.

Therefore, in the experiments described here, we examined the role of the oocyte cytoplasm in the transition of sperm nuclei to metaphase chromosomes. First, we investigated the quantitative relationship between the volume of oocyte cytoplasm and the number of sperm nuclei that could be transformed to metaphase chromosomes, using cell fusion and bisection techniques to increase or decrease the cytoplasmic volume of the oocyte. Secondly, we used the bisection technique to determine whether components associated with the oocyte chromosomes or the nucleoplasm of the GV or the cytoplasm outside the GV were required for the transformation of sperm chromatin to metaphase chromosomes. Thirdly, we tested whether the transition to metaphase required protein synthesized by the oocyte during maturation.

Materials and Methods

Collection and Culture of Ovarian Oocytes

Sexually mature female mice (CD-1, Charles River Canada) were given an injection of 5 IU pregnant mares' serum gonadotropin (Sigma Chemical Co., St. Louis, MO), and killed 44–48 h later. Their ovaries were removed, and fully grown immature oocytes were collected as previously described (9). If the oocytes were to be bisected before germinal vesicle breakdown (GVBD), they were transferred to modified MEM (42), supplemented with 100 µg/ml dibutyryl cyclic AMP (dbcAMP, Sigma Chemical Co.) to prevent spontaneous GVBD (6), and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 2 h. For the other experiments, the oocytes were incubated for 2 h in modified MEM without dbcAMP, to allow GVBD to occur.

Removal of Zonae Pellucidae

After the 2-h incubation in modified MEM, the zona pellucida was removed from all oocytes by exposure to 0.05% α -chymotrypsin (Sigma Chemical Co.) in Dulbecco's medium (15) for 15–30 s at 37°C. Only zona-free oocytes that were completely free of cumulus cells were used in the experiments. Oocytes to be bisected before GVBD were transferred to MEM supplemented by 100 µg/ml dbcAMP. For the other experiments, the oocytes were transferred to modified Whitten's medium (NaCl reduced from 88 to 71 mM) (47).

Bisection of Oocytes

The oocytes were bisected using the procedure developed by Tarkowski (45) and H. Balakier (personal communication). After the α -chymotrypsin treatment, the oocytes were allowed to recover for at least 1 h. The medium holding the oocytes to be bisected after GVBD was supplemented with 5 µg/ml cytochalasin B (Sigma Chemical Co.) (5). The oocytes were transferred to a petri dish (Falcon 1008, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA), whose floor had been lined with agar (1.5% wt/vol; BDH Chemicals Ltd., Toronto) dissolved in protein-free modified L-15 medium (26), that was filled with chilled modified L-15 medium. A glass

needle whose diameter was slightly larger than that of the oocyte was prepared by heating and hand-pulling a micropipette (Drummond microcap, 20 µl; Drummond Scientific Co., Broomall, PA). Then, while observing an oocyte under a dissecting stereomicroscope ($\times 62.5$), the needle was manually lowered onto the oocyte lying on the agar. Under the pressure of the needle, the oocyte was divided into two small spheres, one on each side of the needle, that were connected by a thin membranous strand. This strand was cut using the needle, thus producing two cell fragments. One or both fragments sometimes degenerated within a few minutes of bisection. Those that survived were collected and incubated for 1–2 h. Fragments produced by bisection before GVBD were kept in MEM not containing dbcAMP, and those produced by bisection after GVBD were kept in modified Whitten's medium. Control whole oocytes, which were not bisected, were treated in the same way as the bisected oocytes.

For the bisection experiments, the oocyte fragments and control oocytes were incubated for 18 h after insemination. Then the diameter of each fragment or oocyte was measured using an ocular micrometer and the approximate volume of each cell was calculated. The fragments were separated into groups, according to their volume relative to whole oocytes.

Insemination

Sperm were collected from the vasa deferentia and caudae epididymes of a sexually mature male, and incubated for 1 h to allow capacitation, as previously described (9). The oocytes or fragments were rinsed in F medium, which is a modified Krebs-Ringer solution (22, 33), and transferred in groups of 20–50 to Falcon 3037 dishes containing 0.5 ml of F medium. 5–75 µl of the dilute sperm suspension was added to each of these dishes. The larger volumes were added to the dishes containing fragments, because the fragments were found to be refractory to sperm penetration, as compared with intact oocytes. After the sperm were added, the dishes were gently swirled and returned to the incubator. 45 min later, the oocytes or fragments were transferred to modified Whitten's medium. Uninseminated oocytes were treated in the same way, except that they were not mixed with sperm.

Oocyte Fusion

Inseminated zona-free oocytes were incubated in modified Whitten's medium for 1–2 h and then were exposed for 15–30 s to 0.25% pronase (*Streptomyces griseus*, Calbiochem-Behring Corp., San Diego, CA) dissolved in phosphate-buffered saline. Uninseminated zona-free oocytes were exposed to pronase at the same time. Then all of the oocytes were incubated for 2 h in pronase-free modified Whitten's medium. This brief enzyme treatment increased the frequency of cell fusion; however, the 1–2-h incubation periods in modified Whitten's medium before and after pronase treatment were necessary to prevent cell lysis after the fusion procedure. In preparation for fusion, the oocytes were rinsed in L-15 medium containing 400 µg/ml phytohemagglutinin (Sigma Chemical Co.) (17), so that they could then be aggregated either into doublets, consisting of an uninseminated oocyte and an inseminated oocyte, or into triplets, consisting of an inseminated oocyte between two uninseminated oocytes. The brief exposure to phytohemagglutinin had no observed effect on oocyte behavior. These oocyte aggregates were exposed to polyethylene glycol (PEG, Koch-Light, mol wt 1,450; Terochem Laboratories Ltd., Toronto) for 40–60 s, as described earlier (8), and incubated in modified Whitten's medium for 18 h.

Drug Treatments

Inseminated and uninseminated oocytes at either prometaphase I or metaphase I were transferred to modified Whitten's medium supplemented by 10 µg/ml puromycin (Sigma Chemical Co.) and incubated for 9 h. Previously, we confirmed that puromycin inhibits 99% of protein synthesis reversibly in mouse oocytes (7). In some experiments, the oocytes were then fixed. In other experiments, samples of oocytes were fixed at this time and the rest were transferred to puromycin-free medium and incubated in the presence or absence of 100 µg/ml dbcAMP for an additional 9 h. Then these oocytes were fixed.

Fixation and Staining

The cells were fixed in a fixative consisting of 8 vol ethanol/3 vol water/3 vol acetic acid, mounted on glass slides, and stained using Giemsa, as described previously (7). In some cases, to allow detailed chromosome examination, the living oocytes were exposed for several minutes to a 1% (wt/vol) solution of Na-citrate (BDH Chemicals Ltd.) in water before being fixed (44).

1. *Abbreviations used in this paper:* dbcAMP, dibutyryl cyclic adenosine monophosphate; GV, germinal vesicle; GVBD, germinal vesicle breakdown.

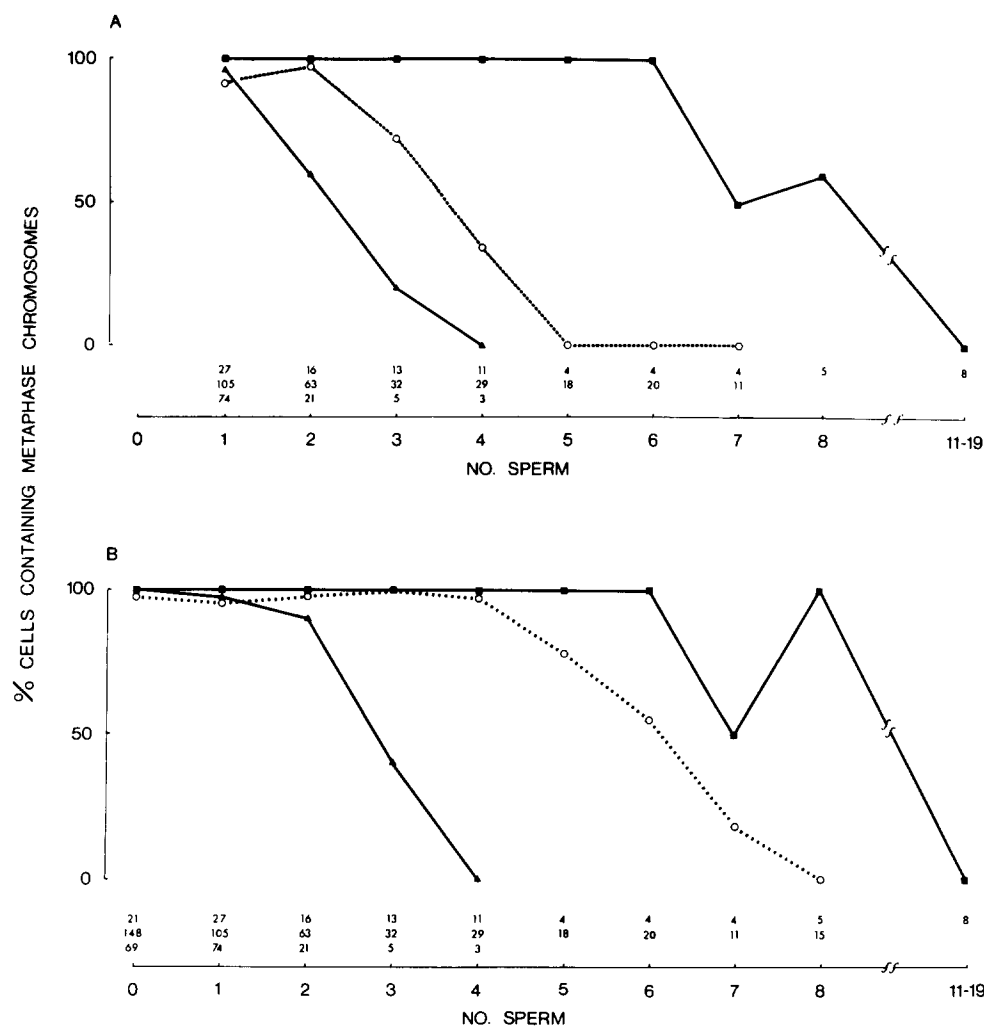


Figure 1. Effect of changes in oocyte cytoplasmic volume on chromosome behavior. Volume was increased by fusion of inseminated oocytes to either one or two uninseminated oocytes. Volume was decreased by bisection of oocytes after GVBD had occurred. The fused and bisected oocytes were incubated for 18 h after insemination and then fixed. Shown for comparison are the results previously obtained using single oocytes (reference 9). The numbers of cells examined are shown along the x axis. (Solid squares) Fused oocytes; (open circles) single oocytes; (solid triangles) oocyte fragments. (A) Percentage of cells containing metaphase sperm chromosomes. (B) Percentage of cells containing metaphase oocyte chromosomes.

Results

Effects of Changes in the Oocyte Cytoplasmic Volume

Giant Oocytes Produced by Cell Fusion. Oocyte fusion began by $\frac{1}{2}$ h after PEG treatment, as judged by the transformation of two or three oocytes into a single giant oocyte. By 3 h after PEG treatment, fusion had occurred in 23%–75% of the aggregates, depending on the batch of oocytes used. When three oocytes had been aggregated, fusion between two of the three occurred ~50% more frequently than did fusion among all three oocytes. Overall, cell fusion occurred in 49% of the aggregates. Thus, the cell fusion technique produced giant oocytes whose volume was either double or triple that of a single oocyte, and which will be referred to as double and triple oocytes, respectively. After overnight incubation, it was observed that numerous small vacuole-like inclusions had developed in most fused oocytes, but in none of the PEG-treated oocytes that remained unfused. This observation suggests that the development of the vacuole-like structures was caused by the fusion of the oocytes, although their origin and influence on the cell activity are unknown.

After the oocytes had been fixed, the sperm nuclei were examined. As shown in Fig. 1 A, single oocytes that have been penetrated by more than four sperm never transform

any of the sperm nuclei into metaphase chromosomes (data from reference 9). However, in contrast to the results observed in single oocytes, it was found that in every fused oocyte penetrated by up to six sperm, all of the sperm nuclei had been transformed into metaphase chromosomes (Figs. 1 A and 2a). Therefore, the addition of cytoplasm to an oocyte substantially increased its capacity to transform sperm nuclei into metaphase chromosomes.

When seven or eight sperm were present in a fused oocyte, all of the nuclei were transformed to metaphase chromosomes in one of four double oocytes and in all five triple oocytes. However, in the other three double oocytes penetrated by seven or eight sperm, as well as in all of the fused oocytes penetrated by 11–19 sperm, none of the sperm nuclei was transformed to metaphase chromosomes (Fig. 1 A). Instead, each nucleus was found as a mass of condensed chromatin in which individual chromosomes could not be distinguished (Fig. 2 b). These results indicate that the maximum number of sperm that could be transformed to metaphase chromosomes is limited to about six in double oocytes and to less than 11 in triple oocytes.

The sperm chromosomes that developed in the oocyte cytoplasm were longer and thinner than the oocyte chromosomes, and each consisted of only one chromatid, as seen in

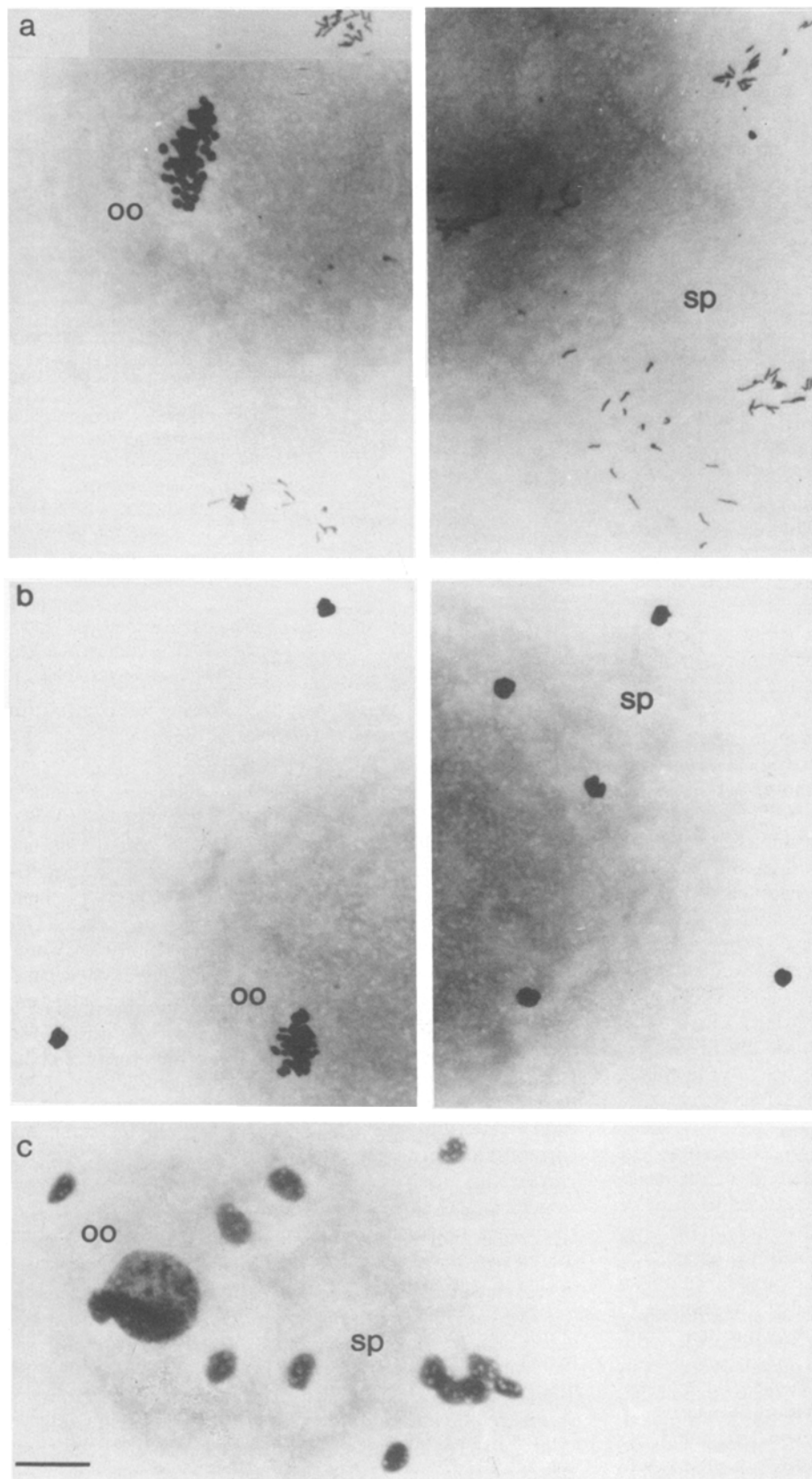


Figure 2. Morphology of sperm and oocyte chromatin in polyspermic fused oocytes. Oocytes at pro-metaphase I were inseminated, fused to either one or two uninseminated oocytes, and incubated for 18 h. Oocyte (oo) and sperm (sp) chromatin have been indicated. (a) Triple oocyte penetrated by six sperm. (b) Double oocyte penetrated by seven sperm. (c) Double oocyte penetrated by 13 sperm. Micrographs *a* and *b* are composites. All micrographs are at the same magnification. Bar, 25 μ m.

Fig. 2 *a*. Usually the sperm chromosomes were found clustered in several groups. Most groups contained 20 chromosomes, which were derived from a single sperm presumably. Some groups contained 40 or 60 chromosomes, sug-

gesting that the chromosomes derived from a few sperm had become mingled together, while other groups contained as few as three chromosomes, indicating that the chromosomes derived from a single sperm did not always remain clustered

together. In 70% of the oocytes, sperm chromosomes were found to be mingled together with the oocyte chromosomes.

The oocyte chromosomes of the fused oocytes also were examined. Previously, it had been observed that, if single oocytes were penetrated by more than six sperm, the oocyte chromosomes did not remain condensed at metaphase but rather became decondensed or coalesced into a small mass (9). In contrast, in the fused oocytes, the oocyte chromosomes remained at metaphase in the presence of at least eight sperm (Figs. 1 *B* and 2 *b*). When double oocytes were penetrated by 11 or more sperm, the oocyte chromosomes became decondensed (Figs. 1 *B* and 2 *c*). In triple oocytes, the degree of polyspermy required to induce oocyte chromosome decondensation is unknown, because none of the oocytes penetrated by more than eight sperm could be definitively classified as triple oocytes. Nevertheless, by comparing the results between the single and fused oocytes, it may be seen that addition of cytoplasm to an oocyte stabilized the metaphase state of its chromosomes.

Oocyte Fragments Produced by Cell Bisection after GVBD. Oocyte fragments produced by bisection of oocytes that had undergone GVBD were separated into two groups, according to whether or not they contained the meiotic spindle. The fragments were inseminated about 2 h after bisection and then incubated for 18 h. Before fixation, each fragment was classified as large or small, according to whether its volume was greater or less than one-third of a whole oocyte.

(a) **Fragments containing oocyte chromosomes.** All of these fragments were larger than one-third of a whole oocyte. As seen in Fig. 1 *A*, almost all of those that had been penetrated by one sperm and two-thirds of those penetrated by two sperm had transformed the sperm nuclei into metaphase chromosomes. In contrast, in the remaining dispermic fragments and in all of the fragments penetrated by three or four sperm, each sperm nucleus was found as a mass condensed chromatin. These chromatin masses morphologically resembled those previously observed in whole oocytes penetrated by more than four sperm (9). These results indicate that the oocyte fragments could transform a maximum of only two sperm nuclei into metaphase chromosomes. Clearly, the reduction in oocyte volume caused a reduction in its capacity to transform sperm nuclei to metaphase chromosomes.

The oocyte chromosomes in the fragments penetrated by up to two sperm were found to be condensed at metaphase II of meiosis. However, in fragments penetrated by three or four sperm, the oocyte chromosomes had become decondensed (Fig. 1 *B*). Thus, penetration of the fragments by three or four sperm could bring about the same effect on the oocyte chromosomes as those brought about by more than six sperm in intact whole oocytes and by more than eight sperm in giant oocytes.

(b) **Fragments lacking oocyte chromosomes.** Table I summarizes the behavior of the sperm nuclei in the fragments that did not contain oocyte chromosomes. Of the large fragments (volume greater than one-third of an oocyte) penetrated by one sperm, 86% transformed the sperm nucleus into metaphase chromosomes. This indicates that this process does not require the presence of the oocyte chromosomes. However, only 23% of the small fragments (volume less than one-third) that were penetrated by one sperm transformed the sperm nucleus to metaphase chromosomes. In

Table I. Chromosome Behavior in Oocyte Fragments

Fragments lacking oocyte chromosomes (bisection after GVBD)					
Appearance of sperm chromatin					
Volume of fragment	No. sperm	No. cases	Metaphase chromosomes	Chromatin mass	Unchanged
>1/3	1	42	36	6	0
	2	15	3	12	0
<1/3	1	40	9	31	0
	2	8	0	8	0

Fragments lacking GV contents (bisection before GVBD)					
Appearance of sperm chromatin					
Volume of fragment	No. sperm	No. cases	Metaphase chromosomes	Chromatin mass	Unchanged
>1/3	1	12	0	9	3
<1/3	1	26	0	22	4
Not recorded	1	40	0	37	3

Oocyte fragments were inseminated and incubated for 18 h. Before fixation, the diameter of each fragment was measured and used to calculate the volume, which is expressed relative to the volume of a single oocyte.

the rest of the small fragments, the sperm nucleus was found as a small mass of condensed chromatin. Therefore, it appears that approximately one-third of the total volume of oocyte cytoplasm is necessary for the transformation of a sperm nucleus into metaphase chromosomes.

Effects of Changing the Quantities of GV and Non-GV Cytoplasmic Materials

Oocytes were bisected before GVBD, producing nucleate and anucleate fragments. Both types of fragments were inseminated and incubated for 18 h. Before fixation, each fragment was classified as large or small, according to its volume. The nucleate fragments contained the same amount of GV nucleoplasm as a whole oocyte, but less non-GV cytoplasmic material than a whole oocyte. Therefore, any observed difference between the results obtained using these fragments and those obtained using whole oocytes must be due to the reduction in the amount of non-GV cytoplasm. At the same time, the results obtained using the anucleate fragments could be used to determine the role of the GV nucleoplasm in sperm chromosome formation.

Nucleate Fragments. Because almost none of the nucleate fragments had a volume less than one-third of a whole oocyte, these fragments were classified according to whether their volumes were greater or less than one-half of a single oocyte. Fig. 3 *A* shows the frequency of sperm chromosome formation among the fragments that contained metaphase oocyte chromosomes. Over 80% of those that were penetrated by one sperm transformed the sperm nucleus into metaphase chromosomes (Fig. 4 *a*). This is about the same frequency that was observed in the control whole oocytes. In contrast, when the fragments were penetrated by more than one sperm, the frequency of transformation to metaphase chromosomes was reduced significantly as compared with the whole oocytes (χ^2 , $P < 0.05$). When two sperm were present in a cell, only 30% of the small fragments transformed the nuclei to metaphase chromosomes, in contrast to 95% of the whole oocytes. When three sperm were present, these were transformed to metaphase chromosomes in none

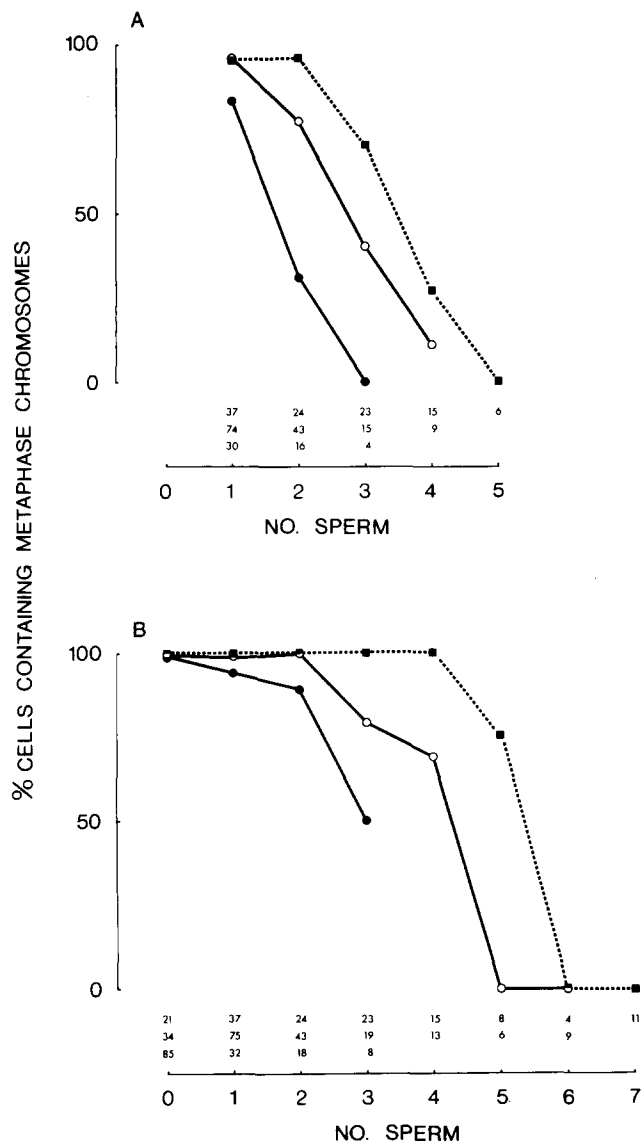


Figure 3. Chromosome behavior in nucleate oocyte fragments produced by bisection before GVBD. Oocytes containing a GV were bisected, and the nucleate fragments were inseminated and incubated for 18 h. Before fixation, the diameter of each fragment was measured and used to calculate the volume. The numbers of oocytes examined are shown along the x axis. (Solid circles) Fragments whose volume was less than one-half of a single oocyte; (solid squares) fragments whose volume was greater than one-half of a single oocyte; (open circles) control single oocytes, treated in the same manner as the fragments, but not bisected. (A) Percentage of cells containing metaphase sperm chromosomes. Fewer fragments than whole oocytes transformed two or three sperm into metaphase chromosomes (χ^2 , $P < 0.05$). (B) Percentage of cells containing metaphase oocyte chromosomes. Fewer fragments than whole oocytes contained metaphase oocyte chromosomes when three, four, or five sperm had penetrated the cell (χ^2 , $P < 0.05$).

of the small fragments, and in just 40% of the large fragments, in contrast to 70% of the whole oocytes. In the rest of the fragments and whole oocytes, which did not transform the sperm nuclei into metaphase chromosomes, the nuclei were found as small masses of condensed chromatin. These results show that a reduction in the amount of non-GV

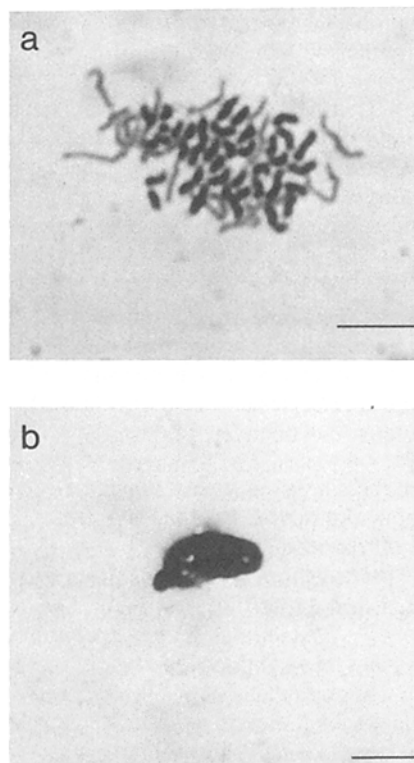


Figure 4. Effect of removing the germinal vesicle on the transformation of the sperm nucleus into metaphase chromosomes. Oocytes containing a GV were bisected into nucleate and anucleate fragments, which were inseminated and incubated for 18 h. (a) Nucleate fragment penetrated by one sperm. The sperm chromosomes are longer, thinner, and more lightly-stained than the oocyte chromosomes. (b) Anucleate fragment penetrated by one sperm. Bar, 10 μ m.

cytoplasmic material decreased the capacity of the oocyte cytoplasm to transform sperm nuclei into metaphase chromosomes.

During the 18-h incubation after bisection, the frequency of maturation to metaphase II in the nucleate fragments was lower than that in the control whole oocytes, even when no sperm were present in the cells (χ^2 , $P < 0.005$, data not shown). This suggests that bisection interfered with the progression of meiosis. In most fragments penetrated by no, one, or two sperm, the oocyte chromosomes were condensed at metaphase I of meiosis (Fig. 3 B). However, in small fragments penetrated by three sperm, and in large fragments penetrated by five or six sperm, the oocyte chromosomes had become decondensed or had coalesced into a darkly stained dot of chromatin. Given that whole oocytes could maintain metaphase oocyte chromosomes in the presence of up to five sperm, a reduction in the amount of non-GV cytoplasmic material had decreased the capacity of the oocyte cytoplasm to maintain the oocyte chromosomes at metaphase.

Anucleate Fragments. In contrast to the results obtained using the nucleate fragments, metaphase sperm chromosomes were never found in the anucleate fragments (Table I), even when these fragments had been treated before fixation with sodium citrate to enhance chromosome resolution (44). Instead, in most fragments, the sperm chromatin had become only slightly dispersed (Fig. 4 b). No particular mor-

phology of the chromatin was observed consistently; rather, its size, shape, and intensity of staining varied among fragments. In some fragments, the sperm nucleus had not undergone any morphologic transformation, but rather retained the typical shape of the sperm head. These results show that in the absence of the GV material, the oocyte cytoplasm was completely deprived of the ability to transform a sperm nucleus into individually separated metaphase chromosomes.

Effects of Inhibition of Protein Synthesis

Oocytes Exposed to Puromycin at Prometaphase I. Oocytes at prometaphase I were inseminated and incubated for 9 h either in the presence of puromycin, which inhibits 99% of protein synthesis in mouse oocytes (7), or in its absence. In the absence of puromycin, most oocytes penetrated by up to three sperm transformed the sperm nuclei into darkly stained arrowhead-shaped masses of chromatin or into metaphase chromosomes during the 9-h incubation (Fig. 5 *a*, Table II), as described previously (9). In contrast, in oocytes treated with puromycin, the sperm were found as larger masses of dispersed chromatin (Fig. 5 *b*, Table II). These results indicate that dispersion of the sperm chromatin within the oocyte cytoplasm could occur in spite of the inhibition of protein synthesis. However, the dispersed chromatin was not further transformed into metaphase chromosomes.

Oocytes Exposed to Puromycin at Metaphase I. Oocytes that had been inseminated at prometaphase I were incubated for 8 h in the absence of puromycin, to allow the oocytes to reach metaphase I and the sperm chromatin to begin its transformation into metaphase chromosomes. Then these oocytes were incubated for 9 h in the presence or absence of puromycin. In the absence of puromycin, oocytes penetrated by up to three sperm completed meiosis to metaphase II and transformed the sperm chromatin to metaphase chromosomes (Fig. 5 *c*, Table II), as described previously (9). In contrast, in the oocytes treated with puromycin, none of the sperm nuclei were transformed into metaphase chromosomes (Fig. 5 *d*, Table II). These results indicate that even when the sperm nucleus had resided in the oocyte cytoplasm for 8 h, protein synthesis had to continue in the oocyte for the sperm nucleus to be transformed to metaphase chromosomes.

The cytological examination of the oocytes treated at metaphase I with puromycin revealed that they contained between one and four interphase nuclei of varying sizes (Fig. 5 *d*, Table III). Previous results had shown that puromycin treatment of oocytes at metaphase I caused the oocyte chromosomes to decondense to form an interphase nucleus (7). Therefore, in the present case, probably one of the nuclei in each oocyte was derived from the oocyte chromosomes. In that uninseminated oocytes rarely developed more than one nucleus when treated with puromycin (Table III) and oocytes penetrated by up to two sperm are not inhibited from first polar body formation (9), it may reasonably be assumed that the other interphase nuclei were derived from sperm chromatin.

From Table II, it may be seen that the oocytes treated with puromycin at prometaphase I could not transform either the oocyte chromosomes or the sperm nuclei into interphase nuclei, whereas the oocytes treated at metaphase I transformed both the oocyte chromosomes and the sperm nuclei into interphase nuclei, regardless of whether the oocytes had been inseminated at prometaphase I or at metaphase I.

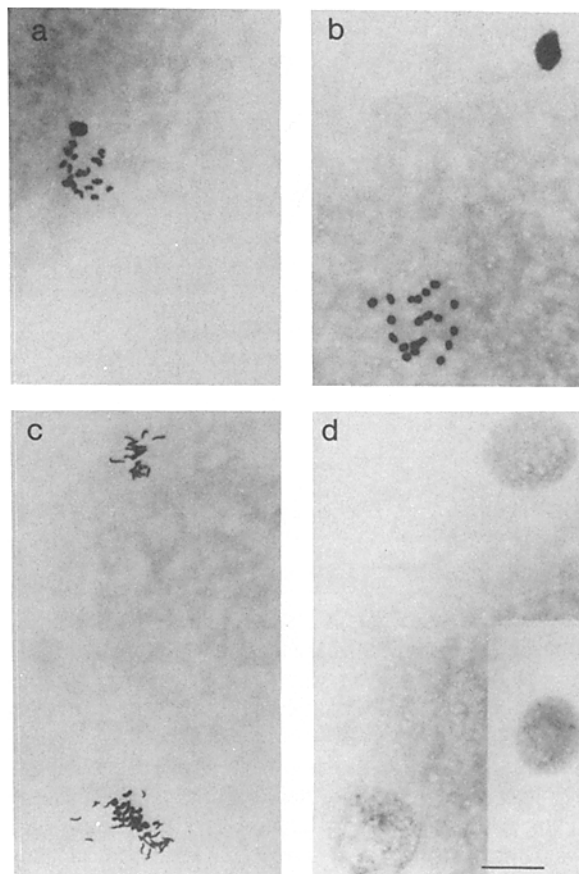


Figure 5. Effect of protein synthesis inhibition on the behavior of the sperm nucleus within the oocyte cytoplasm. Oocytes were inseminated, and incubated in the absence (*a* and *c*) or presence (*b* and *d*) of puromycin. (*a*) Oocyte inseminated at prometaphase I, and incubated for 9 h in the absence of puromycin. Oocyte chromosomes and a condensed mass of sperm chromatin are visible. (*b*) Oocyte inseminated at prometaphase I, and incubated for 9 h in the presence of puromycin. Oocyte chromosomes and a larger mass of sperm chromatin are visible. (*c*) Oocyte inseminated at prometaphase I, and incubated for 17 h in the absence of puromycin. Oocyte chromosomes and two sets of sperm chromosomes, one mingled with the oocyte chromosomes, are visible. (*d*) Oocyte inseminated at prometaphase I, incubated for 8 h (to metaphase I) in the absence of puromycin, and then incubated for 9 h in the presence of puromycin. Three interphase nuclei are visible in the composite micrograph. All micrographs are at the same magnification. Bar, 25 μ m.

Therefore, it appears that the ability of the oocyte cytoplasm to induce transformation of the sperm chromatin to an interphase nucleus develops between prometaphase I and metaphase I, independently of the presence of the sperm chromatin.

Table II also shows that the frequency of sperm transformation to interphase nuclei was reduced when more than one sperm penetrated the oocyte. In these oocytes, the sperm chromatin underwent only a slight dispersion, even though the oocyte's own chromosomes were transformed to an interphase nucleus. Thus, there was a limit to the number of sperm nuclei that could be transformed to interphase nuclei within an oocyte in which protein synthesis had been inhibited at metaphase I.

Reversibility of Puromycin-induced Sperm Nuclear For-

Table II. Morphology of Sperm Chromatin in Oocytes Treated at Prometaphase I or Metaphase I with Puromycin

Stage at insemination	Stage at puromycin treatment	No. sperm	Morphology of sperm chromatin					
			Control			Puromycin-treated		
			Metaphase chromosomes	Dispersed chromatin mass	Interphase nucleus	Metaphase chromosomes	Dispersed chromatin mass	Interphase nucleus
Promet I	promet I	1	7	1	0	2	11	0
		2	1	0	0	0	12	0
		3	2	0	0	0	9	0
Promet I	met I	1	6	1	0	0	5	26
		2	0	0	0	1	10	9
		3	4	0	0	1	2	1
Met I	met I	1	7	1	1	0	4	5
		2	3	1	0	0	0	1
		3	3	0	0	0	4	0

Oocytes were inseminated at prometaphase I (promet I) or metaphase I (met I) of maturation. At the stage indicated in the second-from-left column, they were transferred to medium containing puromycin. Control inseminated oocytes were transferred to puromycin-free medium. 9 h later, the oocytes were fixed.

mation. Previous results (7) had shown that oocyte chromosomes induced to form interphase nuclei by protein synthesis inhibition would return to the metaphase state after protein synthesis was allowed to resume. To see whether sperm-derived interphase nuclei also would form metaphase chromosomes after protein synthesis resumed, the following experiment was performed. Oocytes at prometaphase I were inseminated, incubated to metaphase I, and treated with puromycin. Immediately after puromycin treatment, a sample of oocytes was fixed. Cytological examination revealed that puromycin treatment had caused the sperm chromatin and oocyte chromosomes to form interphase nuclei in all 12 monospermic and 6 of 11 dispermic cases. The rest of the oocytes, which were not fixed, were incubated in puromycin-free medium. 12 h later, metaphase sperm and oocyte chromosomes were found in 16 of 19 monospermic and five of six dispermic oocytes. The remaining four oocytes still contained decondensed sperm and oocyte chromatin. Thus, the sperm chromatin could undergo transition from an interphase state to a metaphase state after protein synthesis resumed.

However, if the oocytes were transferred to medium containing dbcAMP after the puromycin treatment, the sperm and oocyte interphase nuclei were transformed to metaphase chromosomes in only 4 of 10 monospermic and one of seven dispermic oocytes. In the rest of the oocytes, the sperm and oocyte nuclei remained in interphase. Therefore, the transition from interphase to metaphase in the oocytes was inhibited in the presence of dbcAMP, as previously observed (8). These results indicate that the sperm chromatin in the oocyte cytoplasm responds in the same way as the oocyte chromosomes to the effects of a protein synthesis inhibitor and dbcAMP.

Discussion

Relationship between Cytoplasmic Volume and Chromosome Behavior

Previous results showed that when a maturing oocyte of the mouse was penetrated by up to three or four sperm, each sperm nucleus was transformed, without prior development

of a pronucleus, into metaphase chromosomes within the oocyte cytoplasm. However, when an oocyte was penetrated by more than four sperm, none of the nuclei was transformed into metaphase chromosomes, and each instead was found as a small mass of chromatin (9). Thus, there was a limit to the number of sperm that could be transformed to metaphase chromosomes within an oocyte. In the experiments reported here, it was found that when the cytoplasmic volume of an oocyte was increased by cell fusion, up to eight sperm nuclei could be transformed into metaphase chromosomes. Conversely, if the cytoplasmic volume of an oocyte was reduced by bisection after GVBD, no more than two sperm could be transformed into metaphase chromosomes. Thus, the capacity of the oocyte cytoplasm to transform sperm nuclei to metaphase chromosomes was related to its volume. These results suggest that the oocyte cytoplasmic factor which transforms the sperm nucleus into metaphase chromosomes may be stoichiometrically titrated by sperm nuclear material. In fact, most of the cytoplasmic fragments whose volume was less than one-third of a whole oocyte were inhibited from transforming even one sperm nucleus into metaphase chromosomes. This observation, taken together with the fact that a single oocyte can transform a maximum of three or four sperm nuclei to metaphase chromosomes, suggests that an amount of cytoplasm equal to about one-third of a single oocyte contains enough factor to transform one sperm nucleus to metaphase chromosomes.

The results reported here also showed that when fused oo-

Table III. Number of Nuclei That Developed in Oocytes Treated at Metaphase I with Puromycin

Group	No. oocytes	Oocytes				
		No. nuclei after puromycin treatment				
		0	1	2	3	4
		%	%	%	%	%
Uninseminated	90	7	89	4	0	0
Inseminated at prometaphase I	119	13	51	27	8	2
Inseminated at metaphase I	22	18	55	23	5	0

cytes contained more than 11 sperm nuclei, or when oocyte fragments contained more than two sperm, the oocyte chromosomes did not remain condensed at metaphase. Instead, they became either decondensed or coalesced into a darkly stained dot of chromatin. Previously, these morphologic changes in the oocyte chromosomes had been observed in single oocytes penetrated by more than six sperm (9). These results indicate that polyspermy caused an oocyte to lose the ability to keep its own chromosomes condensed at metaphase. Furthermore, the degree of polyspermy required to abolish this ability seems to be related to the volume of cytoplasm in which the sperm reside. This suggests that the oocyte chromosomes may be maintained in a condensed metaphase condition by cytoplasmic factors that are titrated by sperm.

A cytoplasmic activity that induces the transition of chromatin to metaphase has been demonstrated not only in maturing oocytes (see introductory matter), but also in mammalian tissue-culture cells at mitosis (23, 36, 40, 43). This activity induces premature chromosome condensation when an interphase cell is fused to the mitotic cell. It also has occasionally been manifested on the chromatin of sperm that has been fused to the mitotic cell (24). In connection with our results, it is interesting that when several interphase cells are fused to one mitotic cell, chromosome condensation of the interphase nuclei frequently does not occur (21, 37). Thus, the supply of chromosome condensation activity in metaphase tissue-culture cells may be limited, as it is in the experiments described here.

Role of GV and Non-GV Cytoplasmic Materials

A role for oocyte GV components in sperm chromosome formation is evident from the fact that, although most anucleate fragments induced a slight dispersion of the sperm chromatin, transformation of the chromatin into metaphase chromosomes was never observed. Even monospermic fragments whose volume was greater than one-third of a whole oocyte never transformed the sperm nucleus to metaphase chromosomes, indicating that this inability is not due to a quantitative insufficiency of the cytoplasm. Rather, sperm chromosome formation requires components that are associated with the oocyte GV. Because, when the chromosomes of the oocyte were removed by bisection after GVBD, the remaining fragment could induce sperm chromosome formation, these nuclear components are distributed through the cytoplasm after GVBD.

A similar effect of removing the GV on the transformation of nuclei to metaphase chromosomes was observed in amphibian oocytes when brain nuclei or sperm nuclei were injected into maturing oocytes (25, 50). It also was found that proteins that had accumulated in the GV became preferentially bound to the metaphase chromosomes and stabilized them (31).

The role of GV material in the transformation of the sperm nucleus to metaphase chromosomes may be compared to its role in the development of the male pronucleus. GV material is required for male pronuclear development in a variety of animals, including mammals, amphibians, and invertebrates (reviewed in references 27 and 28). In fact, proteins accumulated in the GV later become redistributed to the nuclei of the zygote (14, 16, 35). In addition, it was found that sperm nuclei that were not transformed into pronuclei in enucleated

frog eggs also were not transformed into mitotic chromosomes at the time when mitosis occurred in fertilized nucleated eggs (27). Thus, it appears that GV material is required for transformation of the sperm nucleus either into metaphase chromosomes or into a pronucleus.

The transformation of sperm nuclei to metaphase chromosomes appears also to require non-GV cytoplasmic material. This may be inferred from the observation that the maximum number of sperm that could be transformed to metaphase chromosomes was reduced in the nucleate fragments produced by oocyte bisection before GVBD, in spite of the fact that the entire GV nucleoplasm had been retained in these fragments. Furthermore, in dispermic and trispermic nucleate fragments, the frequency of sperm chromosome formation was higher in the large fragments than in the small fragments, which contained less non-GV cytoplasmic material. These results suggest that the transformation of sperm nuclei to metaphase chromosomes requires non-GV cytoplasmic factors in the oocyte that may be titrated by the sperm nuclei.

Similar results were observed in the frog by Ziegler and Masui (50, 51), who examined the transformation of brain nuclei to metaphase chromosomes in the cytoplasm of the maturing oocyte. They found that this transformation required both GV nucleoplasm and non-GV cytoplasmic materials. Our experiments indicate that the same requirements hold in the mouse.

Role of Protein Synthesis

When the inseminated oocytes were incubated in the presence of puromycin, the sperm chromatin became dispersed in the oocyte cytoplasm. Therefore, the oocyte cytoplasmic factors required for the dispersion of the sperm chromatin appear to develop in the maturing oocyte independently of continuing protein synthesis. The appearance of these factors, which may include thiol groups (33, 38, 41, 48) and negatively charged molecules (11), in the oocyte coincides with GVBD in many species (28).

However, the puromycin-treated oocytes were unable to further transform the dispersed sperm chromatin to metaphase chromosomes, suggesting that protein synthesis is required for this transformation. In maturing oocytes, the transition to metaphase appears to be caused by a cytoplasmic activity known as maturation-promoting factor (30). Maturation-promoting factor activity appears in the cytoplasm shortly before GVBD, disappears near the time of metaphase I, and then reappears after metaphase I. Its reappearance after metaphase I depends on protein synthesis in frogs (18) and starfish (39). In this light, the absence of sperm chromosome formation observed in this study may be due to a failure of maturation-promoting factor to reappear after metaphase I in the cytoplasm of the puromycin-treated oocyte.

In the oocytes whose protein synthesis was inhibited at metaphase I, the sperm chromatin, which failed to form metaphase chromosomes, were transformed instead into structures resembling interphase nuclei. These nuclei morphologically resembled male pronuclei, except that they usually were smaller. However, the number of sperm nuclei that could be transformed to interphase nuclei after protein synthesis inhibition was limited to one or two within one oocyte. In this connection, it is interesting to note that the capacity of fertilized eggs to transform sperm into fully-formed pronuclei also is limited (20, 49).

When the puromycin-treated oocytes were allowed to resume protein synthesis, the interphase nuclei condensed to form metaphase chromosomes. Therefore, the decondensation of sperm chromatin to interphase nuclei induced by protein synthesis inhibition was reversible. However, this transition from interphase to metaphase was inhibited in the presence of dbcAMP, even after protein synthesis had resumed. Similar effects of protein synthesis inhibitors and dbcAMP on oocyte chromosomes were reported previously (8). These similarities imply that the sperm chromatin is influenced by the same cytoplasmic factors that regulate the behavior of the oocyte chromosomes.

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References

- Balakier, H. 1978. Induction of maturation in small oocytes from sexually immature mice by fusion with meiotic or mitotic cells. *Exp. Cell Res.* 112:137-141.
- Balakier, H. 1979. Interspecific heterokaryons between oocytes and blastomeres of the mouse and the bank vole. *J. Exp. Zool.* 209:323-330.
- Balakier, H., and R. Czolowska. 1977. Cytoplasmic control of nuclear maturation in mouse oocytes. *Exp. Cell Res.* 131:137-141.
- Balakier, H., and A. Tarkowski. 1980. The role of the germinal vesicle karyoplasm in the development of the male pronucleus in the mouse. *Exp. Cell Res.* 128:79-85.
- Barton, S. C., and M. A. H. Surani. 1983. Microdissection of the mouse egg. *Exp. Cell Res.* 146:187-191.
- Cho, W. K., S. Stern, and J. D. Biggers. 1974. Inhibitory effect of dibutyl cyclic AMP on mouse oocyte maturation in vitro. *J. Exp. Zool.* 187:383-386.
- Clarke, H. J., and Y. Masui. 1983. The induction of reversible and irreversible chromosome decondensation by protein synthesis inhibition during meiotic maturation of mouse oocytes. *Dev. Biol.* 97:291-301.
- Clarke, H. J., and Y. Masui. 1985. Inhibition by dibutyl cyclic AMP of the transition to metaphase of mouse oocyte nuclei and its reversal by cell fusion to metaphase oocytes. *Dev. Biol.* 108:32-37.
- Clarke, H. J., and Y. Masui. 1986. Transformation of sperm nuclei to metaphase chromosomes in the cytoplasm of maturing oocytes of the mouse. *J. Cell Biol.* 102:1039-1046.
- Czolowska, R., J. A. Modlinski, and A. Tarkowski. 1984. Behaviour of thymocyte nuclei in nonactivated and activated mouse oocytes. *J. Cell Sci.* 69:19-34.
- Dean, J. 1983. Decondensation of mouse sperm chromatin and reassembly into nucleosomes mediated by polyglutamic acid in vitro. *Dev. Biol.* 99:210-216.
- DiBerardino, M. A. 1980. Genetic stability and modulation of metazoan nuclei transplanted into eggs and zygotes. *Differentiation*. 17:17-30.
- Donahue, R. P. 1968. Maturation of the mouse oocyte in vitro. I. Sequence and timing of nuclear progression. *J. Exp. Zool.* 169:237-250.
- Dreyer, C., H. Singer, and P. Hausen. 1981. Tissue specific nuclear antigens in the germinal vesicle of *Xenopus laevis* oocytes. *Roux's Arch. Dev. Biol.* 190:197-207.
- Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis virus. *J. Exp. Med.* 99:167-182.
- Ecker, R. E., and L. D. Smith. 1971. The nature and fate of *R. pipiens* oocyte proteins synthesized during maturation and early cleavage. *Dev. Biol.* 24:559-576.
- Eglitis, M. A. 1980. Formation of tetraploid mouse blastocysts following blastomere fusion with polyethylene glycol. *J. Exp. Zool.* 213:209-213.
- Gerhart, J., M. Wu, and M. W. Kirschner. 1984. Cell cycle dynamics of an M-phase-specific cytoplasmic factor in *Xenopus laevis* oocytes and eggs. *J. Cell Biol.* 98:1247-1255.
- Gurdon, J. B. 1968. Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J. Embryol. Exp. Morphol.* 20:401-414.
- Hirao, Y., and R. Yanagimachi. 1979. Development of pronuclei in polyspermic eggs of the golden hamster. *Zool. Mag. (Tokyo)*. 88:24-33.
- Ikeuchi, T., M. Sanbe, H. Weinfeld, and A. A. Sandberg. 1971. Induction of nuclear envelopes around metaphase chromosomes after fusion with interphase cells. *J. Cell Biol.* 51:104-115.
- Inoue, M., and D. P. Wolf. 1974. Comparative solubility properties of the zona pellucidae of unfertilized and fertilized mouse ova. *Biol. Reprod.* 11:558-565.
- Johnson, R. T., and P. N. Rao. 1970. Mammalian cell fusion: Induction of premature chromosome condensation in interphase nuclei. *Nature (Lond.)*. 226:717-722.
- Johnson, R. T., P. N. Rao, and H. D. Hughes. 1970. Mammalian cell fusion. III. A HeLa cell inducer of premature chromosome condensation active in cells from a variety of animal species. *J. Cell. Physiol.* 76:151-158.
- Katagiri, C., and M. Moriya. 1976. Spermatozoan response to the toad egg matured after removal of the germinal vesicle. *Dev. Biol.* 50:235-241.
- Leibowitz, A. 1963. The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *Am. J. Hyg.* 78:173-180.
- Lohka, M. J., and Y. Masui. 1983. The germinal vesicle material required for sperm pronuclear formation is located in the soluble fraction of egg cytoplasm. *Exp. Cell Res.* 148:481-491.
- Longo, F. J. 1985. Pronuclear events during fertilization. In *Biology of Fertilization*. Vol. 3. C. B. Metz and A. Monroy, editors. Academic Press, Inc., Orlando, FL. 251-298.
- Masui, Y., and H. J. Clarke. 1979. Oocyte maturation. *Int. Rev. Cytol.* 57:185-282.
- Masui, Y., and C. L. Markert. 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* 177:129-145.
- Masui, Y., P. G. Meyerhof, and D. H. Ziegler. 1979. Control of chromosome behaviour during progesterone-induced maturation of amphibian oocytes. *J. Steroid Biochem.* 11:715-722.
- Masui, Y., M. J. Lohka, and E. K. Shibuya. 1984. Roles of Ca ions and ooplasmic factors in the resumption of metaphase-arrested meiosis in *Rana pipiens* oocytes. *Symp. Soc. Exp. Biol.* 34:44-66.
- Miller, M. A., and Y. Masui. 1982. Changes in the stainability and sulphhydryl level in the sperm nucleus during sperm-oocyte interaction in mice. *Gamete Res.* 5:167-179.
- Moriya, M., and C. Katagiri. 1976. Microinjection of toad sperm into oocytes undergoing maturation division. *Dev. Growth Differ.* 18:349-356.
- Motlik, J., V. Kopečný, J. Pivko, and J. Fulka. 1980. Distribution of proteins labelled during meiotic maturation in rabbit and pig eggs at fertilization. *J. Reprod. Fertil.* 58:415-419.
- Nelkin, B., C. Nichols, and B. Vogelstein. 1980. Protein factors from mitotic CHO cells induce meiotic maturation in *Xenopus laevis* oocytes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 109:233-238.
- Obara, Y., L. S. Chai, H. Weinfeld, and A. A. Sandberg. 1974. Prophase of interphase nuclei and induction of nuclear envelopes around metaphase chromosomes in HeLa and Chinese hamster homo- and heterokaryons. *J. Cell Biol.* 62:104-113.
- Perreault, S. D., R. A. Wolff, and B. R. Zirkin. 1984. The role of disulfide bond reduction during mammalian sperm nuclear decondensation in vivo. *Dev. Biol.* 101:160-167.
- Picard, A., G. Peaucellier, F. LeBouffant, C. LePeuch, and M. Dorée. 1985. Role of protein synthesis and proteases in production and inactivation of maturation-promoting activity during meiotic maturation of starfish oocytes. *Dev. Biol.* 109:311-320.
- Rao, P. N., and R. T. Johnson. 1970. Mammalian cell fusion: Studies on the regulation of DNA synthesis and mitosis. *Nature (Lond.)*. 225:159-164.
- Rodman, T. C., F. H. Pruslin, H. P. Hoffmann, and V. G. Allfrey. 1981. Turnover of basic chromosomal proteins in fertilized eggs—a cytoimmunochemical study of events in vitro. *J. Cell Biol.* 90:351-361.
- Schroeder, A. C., and J. J. Eppig. 1984. The developmental capacity of mouse oocytes that matured spontaneously in vitro is normal. *Dev. Biol.* 102:493-497.
- Sunkara, P. S., D. A. Wright, and P. N. Rao. 1979. Mitotic factors from mammalian cells induce germinal vesicle breakdown and chromosome condensation in amphibian oocytes. *Proc. Natl. Acad. Sci. USA*. 76:2799-2802.
- Tarkowski, A. K. 1966. An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics*. 5:394-400.
- Tarkowski, A. K. 1977. In vitro development of haploid mouse embryos produced by bisection of one-cell fertilized eggs. *J. Embryol. Exp. Morphol.* 38:187-202.
- Tarkowski, A. K. 1980. Fertilization of nucleate and anucleate egg fragments in the mouse. *Exp. Cell Res.* 128:73-77.
- Whitten, W. K. 1971. Nutrient requirements for culture of pre-implantation embryos in vitro. *Adv. Biosci.* 6:129-139.
- Wiesel, S., and G. A. Schultz. 1981. Factors which may affect removal of protamine from sperm DNA during fertilization in the rabbit. *Gamete Res.* 4:25-34.
- Witkowska, A. 1981. Pronuclear development and the first cleavage division in polyspermic mouse eggs. *J. Reprod. Fertil.* 62:493-498.
- Ziegler, D. H., and Y. Masui. 1973. Control of chromosome behavior in amphibian oocytes. I. The activity of maturing oocytes inducing chromosome condensation in transplanted brain nuclei. *Dev. Biol.* 35:283-292.
- Ziegler, D., and Y. Masui. 1976. Control of chromosome behavior in amphibian oocytes. II. The effect of inhibitors of RNA and protein synthesis on the induction of chromosome condensation. *J. Cell Biol.* 68:620-628.