

Mechanism of polar body formation in the mouse oocyte: an interaction between the chromosomes, the cytoskeleton and the plasma membrane*

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SUMMARY

The influence of mouse oocyte chromosomes on their immediate environment has been investigated following their dispersal by dissolution of the metaphase spindle with nocodazole. Small clusters of chromosomes become redistributed around the egg cortex in a microfilament-dependent process. Each cluster has the capacity, on removal from nocodazole, to organize a spindle that rotates to yield a polar body. In this process of spindle formation, the chromosome clusters are able both to promote tubulin polymerization in their vicinity and to recruit microtubule-organizing centres (MTOCs) which organize the polymerized tubulin into spindles. In addition each oocyte chromosome cluster, as well as the non-dispersed sperm-derived haploid group of chromosomes, induces a focal accumulation of subcortical actin (corresponding to a filamentous area devoid of organelles) and a loss of surface Concanavalin A binding activity (corresponding to a loss of surface microvilli) in the overlying cortex. This induction ceases with the formation of pronuclei whether or not the pronuclei migrate centrally. Pronuclear formation is sensitive to the action of nocodazole for up to 2–4 h postinsemination, and pronuclear migration is totally sensitive to the drug. If pronuclei are blocked in a peripheral location by nocodazole they are associated with an elevation in Con A binding activity of the overlying membrane which corresponds to an area of the surface rich in blebby microvilli.

INTRODUCTION

The first few hours after fertilization of the mouse oocyte are characterized by major changes in cytoskeletal organization involving both microfilaments (Maro, Johnson, Flach & Pickering, 1984) and microtubules (Maro, Howlett & Webb, 1985; Schatten, Simerly & Schatten, 1985). These changes are associated with the reestablishment of a diploid state, and include sperm incorporation, ejection of the second polar body and formation and migration of pronuclei. The resumption

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of meiosis results in the formation of two very different cells each possessing the same maternal DNA content: a very large cell, the egg, and the polar body which is about 1% the size of the egg. This unequal division retains the bulk of the constituents synthesized during oogenesis in the egg for use subsequently during early embryogenesis. Very little is known about the mechanisms involved in polar body formation. We reported previously on the close association between the cytocortex and both the meiotic female and newly introduced male chromosomes, and demonstrated that in both cases the overlying membrane lacked microvilli (as assessed by a reduction in Concanavalin A binding and by electron microscopic analysis; Eager, Johnson & Thurley, 1976; Johnson, Eager, Muggleton-Harris & Graves, 1975; Shalgi, Phillips & Kraicer, 1978) and was associated with a subcortical focus of stable polymerized actin (Maro *et al.* 1984; Longo & Chen, 1985; Karasiewicz & Soltynska, 1985; Van Blerkom & Bell, 1986; see also Fig. 1E-H). We suggested that the chromosomes, or some factor closely associated with them, might have induced these modifications of the cell cortex. We now present evidence that supports this proposal and suggests that when pronuclei form, the influence of the chromatin on the overlying cytocortex is lost. We also demonstrate two other types of interaction between the chromosomes and the cytoskeleton, namely their ability to promote tubulin polymerization in their vicinity and their ability to recruit cytoplasmic MTOCs in order to form a spindle. Taken together these three properties of the chromosomes provide a basis for explaining how polar body extrusion occurs.

MATERIALS AND METHODS

Recovery and fertilization of oocytes

A sperm suspension was prepared from the cauda epididymides of male HC-CFLP mice (Hacking and Churchill). Two epididymides were immersed in a 0.5 ml drop of Whittingham's medium (Whittingham, 1971) containing 30 mg ml⁻¹ bovine serum albumin (BSA, Sigma) under liquid paraffin (BDH), which had been equilibrated overnight at 37°C in 5% CO₂ in air. The sperm were released into suspension and left to capacitate for 1.5 h at 37°C.

Oocytes were recovered from 3- to 5-week-old (C57B1.10×CBA) F₁ or MF1 mice after superovulation with 5 i.u. pregnant mares' serum (PMS, Intervet) followed 48 h later by 5 i.u. human chorionic gonadotrophin (hCG, Intervet). The females were killed 12.5 h post hCG and the ovulated oocytes released from the oviducts into preequilibrated drops of Whittingham's medium containing 30 mg ml⁻¹ BSA. At 13.5 h post hCG, the sperm suspension was mixed and diluted 1:9 in the drops containing the oocytes, giving a final sperm concentration of 1–2×10⁶ ml⁻¹.

Eggs that were cultured for more than 4 h were removed from the spermatozoa and washed two or three times in preequilibrated medium 16 (Whittingham & Wales, 1969) containing 4 mg ml⁻¹ BSA. The eggs were then cultured further in this medium until harvesting. Unfertilized oocytes were treated in the same way, simply omitting addition of the sperm suspension.

Harvesting of eggs and oocytes for analysis

Oocytes and eggs were freed of their cumulus cells by brief exposure to 0.1 M-hyaluronidase (Sigma), and all fertilized eggs and oocytes were freed from their zonae pellucidae by brief

exposure to acid Tyrode's solution (Nicolson, Yanagimachi & Yanagimachi, 1975), followed by a rinse in medium 2 (M2; Fulton & Whittingham, 1978) containing 4 mg ml^{-1} BSA.

Staining of unfixed cells with Concanavalin A

Zona-free cells were incubated for 5 min at room temperature in FITC-Concanavalin A (FITC-Con A, $700 \text{ } \mu\text{g ml}^{-1}$; Miles) or tetramethylrhodamine-labelled succinyl Concanavalin A (TRMTC-S-Con A, $500 \text{ } \mu\text{g ml}^{-1}$; Polysciences) and rinsed through three changes of M2+BSA.

Cell fixation and immunocytochemical staining

Cells were then placed in specially designed chambers as described previously (Maro *et al.* 1984) except that the chambers were coated first with a solution of 0.1 mg ml^{-1} Concanavalin A (Con A) or, when the cells had been first stained with FITC-Con A or TRMTC-S-Con A, with a 1/20 dilution of Gibco stock PHA.

The cells were then fixed in one of two ways:

(i) For tubulin, actin and lamin staining with either mouse anti-tubulin monoclonal antibody (Amersham), affinity-purified rabbit anti-actin polyclonal antibodies (Gounon & Karsenti, 1981), or human anti-lamin serum (McKeown, Tuffanelli, Fukuyama & Kirschner, 1983) cells were fixed for 30–45 min at 37°C with 3.7% formaldehyde (BDH) in phosphate-buffered saline (PBS), washed in PBS then extracted for 10 min in 0.25% Triton X-100 (Sigma) and washed in PBS.

(ii) For MTOC staining with a human anti-pericentriolar material (PCM) serum (Calarco-Gillam *et al.* 1983) cells were extracted for 10 min in HPEM buffer (10 mM-EGTA, 1 mM- MgCl_2 , 60 mM-PIPES, 25 mM-HEPES, pH 6.9; Schliwa, Euteneuer, Bulinsky & Izant, 1981) containing 0.25% Triton X-100, washed in HPEM buffer and fixed for 30 min with 3.7% formaldehyde in HPEM buffer.

Immunocytochemical staining was performed as described in Maro *et al.* (1984). In order to stain condensed chromosomes, fixed cells were incubated in Hoechst dye 33258 ($5 \text{ } \mu\text{g ml}^{-1}$ in PBS) for 20 min at 20°C .

Photomicroscopy

The coverslips were removed from the chambers and samples were mounted in 'Citifluor' (City University, London) to reduce fading of the reagents and viewed on a Leitz Ortholux II microscope with filter sets N2 for rhodamine-labelled reagents, L2 for fluorescein-labelled reagents and A for Hoechst dye. Photographs were taken on Kodak Tri-X film using a Leitz Vario-Orthomat photographic system.

The three-dimensional structure of the cell is preserved in the whole mount, but as the size of the cells is large ($70 \text{ } \mu\text{m}$ in diameter), it is impossible to photograph the whole cell in the same focal plane. Therefore, in most figures, optical sections showing only one plane through the cell are shown in sharp focus; in some figures optical sections at different focal planes are illustrated.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the surface of the specimens. The procedure used was essentially that of Johnson & Ziomek (1982) as modified in Maro & Pickering (1984). Eggs were examined in a Jeol ISM-35CF microscope under 20 kV.

Transmission electron microscopy

Oocytes and eggs were fixed in 3% glutaraldehyde in 0.1 M-cacodylate buffer pH 7.3 for 30 to 60 min at room temperature and washed twice in the same buffer. They were then postfixed, embedded and sectioned as described in Fleming, Warren, Chisolm & Johnson (1984). Sections were viewed in a Philips 600 electron microscope at 80 kV.

Drugs

A stock solution of 1 mg ml⁻¹ cytochalasin D (CCD, Sigma) in dimethylsulphoxide, a stock solution of 10 mM-taxol (Lot T-4-112, N.I.H., Bethesda, USA) in dimethylsulphoxide and a stock solution of 10 mM-nocodazole (Aldrich) in dimethylsulphoxide stored at -20°C were used in these experiments.

RESULTS

Dispersal of oocyte-derived chromosomes by spindle disruption

Oocytes were placed in medium either with or without the microtubule inhibitor nocodazole (Hoebeke, Van Nigen & De Brabander, 1976), inseminated with spermatozoa 1 h later and then cultured for up to 8 h before staining the chromatin with Hoechst dye 33258. Spermatozoa fertilized oocytes readily, whether or not nocodazole was present, and the sperm chromatin could be distinguished from that of the oocyte by its sharply defined boundary, by its association with the remnants of the sperm tail and, in controls, with the fertilization cone. In control eggs, spindle rotation and polar body extrusion occurred within 2 h (Fig. 1A-D), leaving discrete haploid masses of sperm and oocyte chromosomes within the egg (Fig. 1E,F). In contrast, oocytes incubated in nocodazole underwent spindle dissolution, as assessed both by differential interference contrast microscopy and staining with an anti-tubulin antibody (compare Fig. 1I with 1A) and in most eggs the oocyte-derived chromosomes were dispersed among two to six discrete clumps of variable size located around the egg cortex (Figs 1N, 2). Polar body extrusion could not of course occur in any egg treated continuously with nocodazole (Fig. 1M) and the formation of the fertilization cone at the site of sperm entry was also prevented.

Fig. 1. (A,B) Unfertilized oocyte at second meiotic metaphase double stained for tubulin (A) and chromatin (B).

(C,D) Egg 2 h postinsemination double stained for tubulin (C) and chromatin (D). Note spindle rotation and separation of the two haploid oocyte chromosome masses.

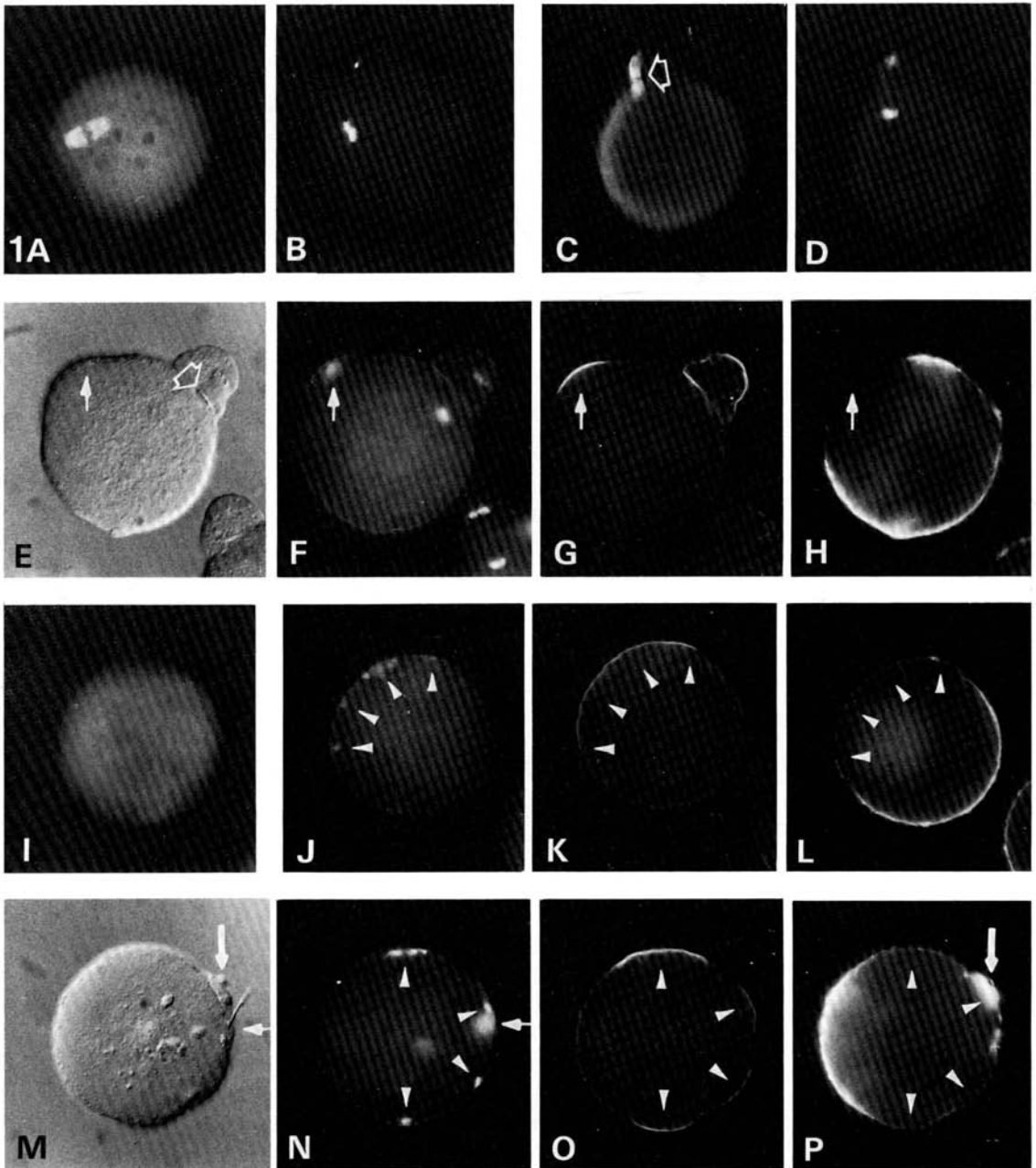
(E,H) Egg 2 h postinsemination triple stained for chromatin (F), actin (G) and Con A (H). Note sperm entry point (arrow) and spindle (arrowhead) in the differential interference contrast (DIC) picture (E) which are associated with cortical actin (G) and loss in Con A binding (H).

(I) Unfertilized oocyte treated for 3 h with 10 µM-nocodazole and stained for tubulin. Note absence of spindle, compare with Fig. 1A.

(J-L) Unfertilized oocyte treated for 3 h with 10 µM-nocodazole and triple stained for chromatin (J), actin (K) and Con A (L). Note chromosome clusters dispersed (arrowheads) around the cortex and associated with cortical actin (K) and loss of Con A binding (L).

(M-P) Egg treated with 10 µM-nocodazole for 1 h prior to insemination and 6 h postinsemination and then triple stained for chromatin (N), actin (O) and Con A (P). Note sperm entry point (small arrow) and degenerating first polar body (large arrow) which binds Con A strongly (P). Oocyte chromosome clusters are dispersed (arrowheads) round the cortex and associated with cortical actin (O) and loss of Con A binding (P) as is the sperm-derived chromatin (N, arrow, slightly off the plane of focus). ×320.

The process of chromosome dispersal appears to depend upon a functional microfilament system since the presence of cytochalasin D (CCD) in addition to nocodazole over the first hour postinsemination blocked dispersal completely (Fig. 2). However, the dispersal of the chromosomes does not depend upon fertilization, since unfertilized oocytes incubated for up to 8 h in nocodazole also showed evidence of chromosome dispersal (Fig. 1J, 2), although the dispersal



process may be slower than in fertilized eggs (Fig. 2). Moreover addition of nocodazole to ageing unfertilized oocytes (i.e. equivalent to between 2 and 6 h postinsemination of fertilized eggs) still resulted in dispersal (data not shown). In contrast, if oocytes were inseminated in control medium and then transferred to nocodazole at 2 h or later, dispersal of oocyte chromosomes did not occur (data from 171 oocytes not shown). During these first 2 h postinsemination, anaphase, telophase, spindle rotation and polar body formation takes place (Maro *et al.* 1984).

From these results we conclude that (i) an intact spindle is required for the integrity of the oocyte-derived chromosome masses, (ii) in the absence of a spindle, oocyte chromosomes disperse around the cortex *via* a microfilament-mediated process, (iii) this dispersal process is not dependent on activation at fertilization, (iv) in fertilized eggs which have passed beyond metaphase before

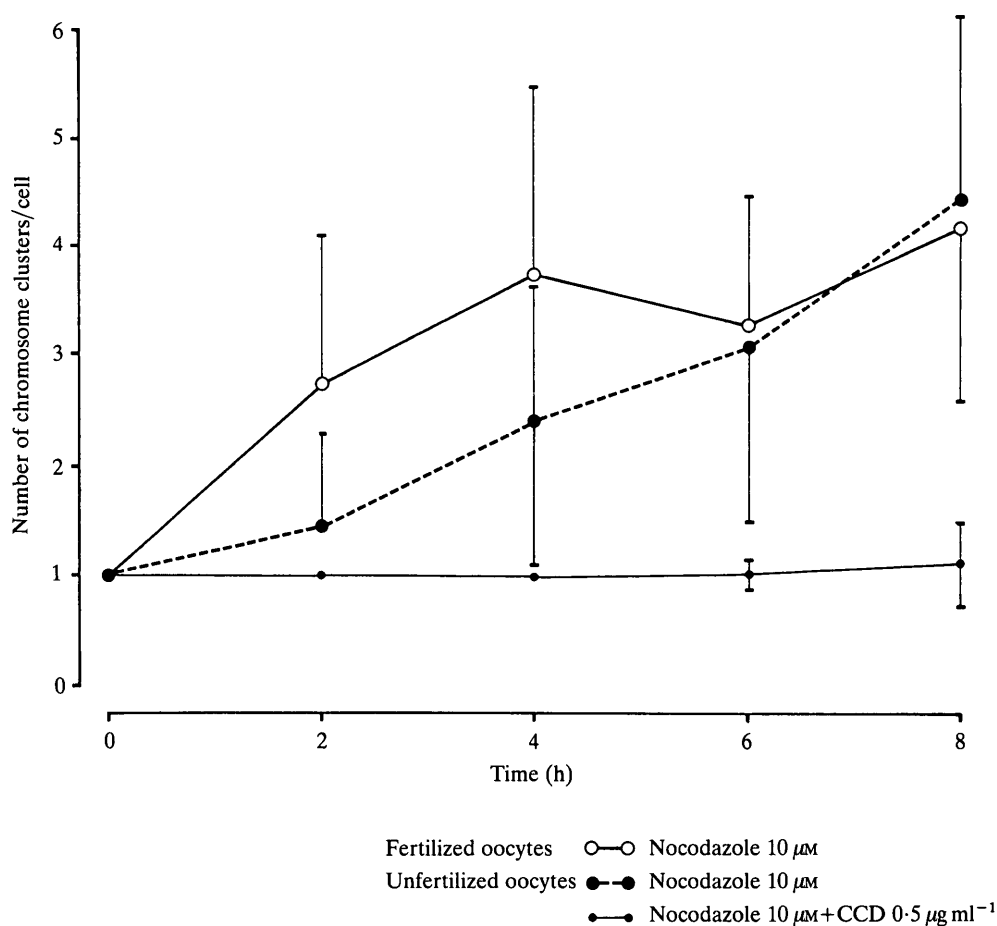


Fig. 2. Dispersal of oocyte chromosomes in mouse oocytes treated with nocodazole. The number of cells examined was 234 for the unfertilized oocytes treated with nocodazole, 123 for the unfertilized oocytes treated with nocodazole and CCD, and 154 for the oocytes fertilized in the presence of nocodazole (mean \pm s.d.).

addition of nocodazole, the chromosomes do not disperse. We next examined the capacity of dispersed chromosomes to affect the cytocortical organization of the egg.

Cytocortical changes in nocodazole-treated eggs

In the continuing presence of nocodazole, a focus of bright subcortical actin staining and an area lacking Con A binding sites developed adjacent to the clump of sperm chromosomes and at almost all sites of dispersed oocyte chromosomes in both fertilized and unfertilized oocytes (Table 1; Fig. 1J–P). The area of cortex affected at each site was often extensive, and coalescence of adjacent areas often occurred (compare Fig. 1K and 1O). Only when very small clusters of oocyte chromosomes were examined was this association not detected. These foci of actin and reduced Con A binding persisted in the presence of nocodazole, but were lost in control eggs when pronuclei formed and migrated centrally.

When nocodazole-treated eggs were examined on the scanning electron microscope (SEM) they were found to have either an increase in the size of the area devoid of microvilli or the presence of more than one of these areas (Fig. 3A–C; Table 2). Using transmission electron microscopy, an electron-dense area was observed in the cytocortex overlying the chromosomes and extending in a wide area around them (Fig. 3E). This electron-dense area was seen at higher magnification to be rich in filamentous structures (Fig. 3F). Very few organelles were found in this area of the cytocortex while many were observed on the cytoplasmic side of the chromosomes (Fig. 3E) or in other regions of the cell cortex remote from the chromatin (Fig. 3D). No microvilli were observed in the area of the cell surface adjacent to the chromosomes (Fig. 3E) while many of them were observed in other regions of the cell surface (Fig. 3D), confirming the SEM data.

From these results we conclude that chromosome clusters induce in their vicinity (i) a disappearance of Con A binding sites and of microvilli at the cell surface, (ii) an actin-rich filamentous area in the cell cortex between the chromosomes and the plasma membrane. We next examined whether each clump of dispersed chromosomes could also engage in polar body formation.

Multiple polar body formation in nocodazole-treated eggs after removal of the drug

Eggs were fertilized *in vitro* in the presence of nocodazole, then removed from the drug after 2 h or later and cultured in control medium to 8 h before analysis. Despite removal from the drug, the dispersal of the oocyte-derived chromosomes nonetheless persisted (Table 3). Moreover, many eggs showed formation of multiple spindles that were fully or partially rotated, or of multiple polar bodies, each associated with a cluster of oocyte-derived chromosomes but never with sperm-derived chromosomes (Fig. 4A–E; Table 3). The proportion of eggs with polar bodies formed or forming was higher when drug removal occurred at 2 or 4 h than at 6 h. These polar bodies were forming in the cortical area rich in

Table 1. *Cortical changes induced by oocyte-derived chromosome clusters in nocodazole-treated oocytes*

Incubation conditions (37°C)	Number of cells examined	% of oocytes with given number of oocyte chromosome clusters			% of oocytes where oocyte chromosomes are associated with an area:	
		1	2	>2	Devoid of Con A binding sites	Rich in cortical actin
Control						
2 h	10	30	70		100	100
4 h	32	100			78	56
6 h	27	100			18	11
8 h	95	100			2	
Nocodazole 10 μ M						
2 h	35	25	17	58	97	66
4 h	20	5	25	70	100	100
6 h	40	7	20	73	98	100
8 h	59	4	18	78	97	100

microfilaments and devoid of microvilli as do normal polar bodies (Fig. 4F–K). Not all chromosome clusters were associated with spindles, but most were.

From these results we conclude that (i) the clumps of dispersed oocyte chromosomes have the capacity to induce the formation of a spindle that can rotate and yield a polar body, (ii) the sperm-derived chromosomes lack this capacity, and (iii) the capacity to form spindles and polar bodies persists in nocodazole-treated fertilized oocytes well beyond the 2 h period within which these processes are completed normally in control fertilized oocytes.

In order to form a polar body, both microtubules and microtubule-organizing centres (MTOCs) are required to organize a spindle. Therefore, we next examined whether these were present in the vicinity of dispersed chromosomes.

Chromosomes and microtubule-organizing centres (MTOCs)

In the mouse oocyte, the MTOCs do not have associated centrioles (Szollosi, Calarco & Donahue, 1972; Calarco-Gillam *et al.* 1983), instead multiple foci of

Fig. 3. (A–C) Scanning electron microscopy of mouse oocytes.

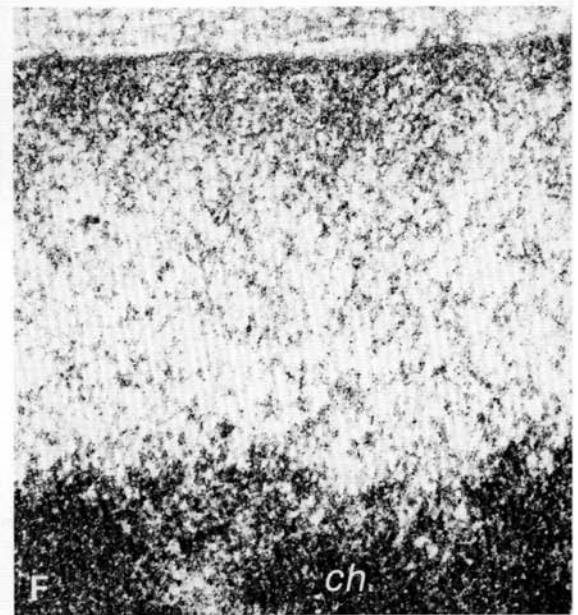
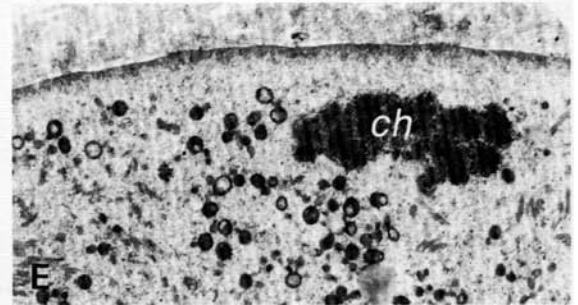
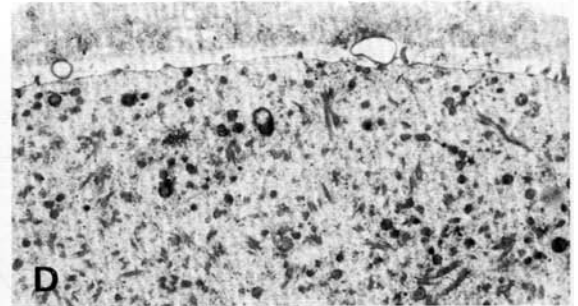
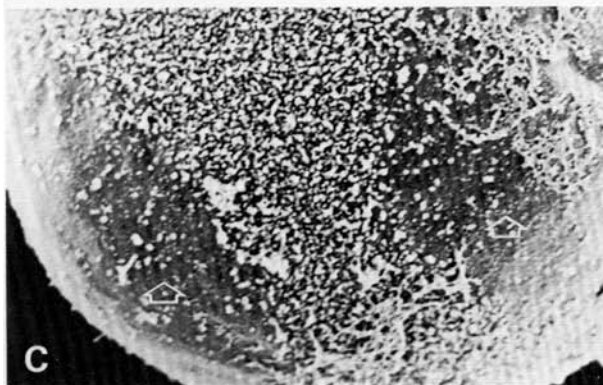
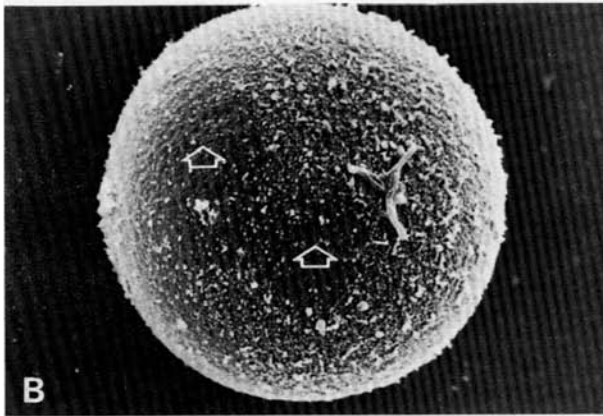
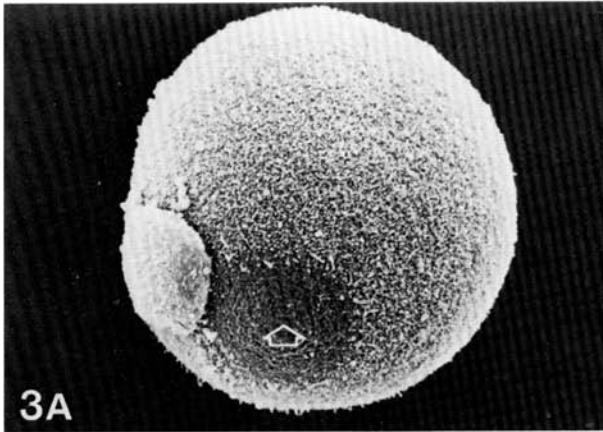
(A) Control oocyte, 20 h post hCG. Note area free of microvilli (open arrow) adjacent to the first polar body, reflecting the presence of the underlying second meiotic spindle. $\times 1200$.

(B) Oocyte treated with 10 μ M-nocodazole for 6 h. Note two areas free of microvilli (open arrows). $\times 1200$.

(C) Oocyte treated with 10 μ M-nocodazole for 6 h. Note two areas free of microvilli (open arrows) and the boundary of microvilli between these two areas. $\times 2400$.

(D–F) Transmission electron microscopy of an oocyte treated with 10 μ M-nocodazole for 6 h. *ch*, chromosomes. Note filamentous area devoid of organelles between the chromosomes and the plasma membrane and extending beyond the immediate confines of the chromosomes (E,F). In contrast organelles can be found in the vicinity of the plasma membrane in region of the cell remote from the chromosomes (D). $\times 6500$ (D,E), $\times 52000$ (F).

pericentriolar material (PCM) can be observed at the poles of the spindle. In addition, many discrete foci of PCM are dispersed throughout the subcortical cytoplasm of the unfertilized oocyte (Maro *et al.* 1985). When unfertilized oocytes were treated for 6 h with nocodazole and then double stained with an anti-PCM serum and Hoechst dye, very few chromosome clusters were found associated with a PCM focus (21 out of 348; 6%; Fig. 5). However, after removal of the drug, spindle formation occurred within 1 h in a few cases and within 3 h in most cases



and PCM could be observed at the spindle poles (Fig. 6). We must note that the dispersion of the PCM foci seems also to be a microfilament-dependent process since 83 % of the polar PCM foci remained associated with the chromosomes in oocytes treated for 6 h with both nocodazole and cytochalasin D (data from 21 oocytes not shown).

From these results we conclude that (i) in the absence of microtubules, PCM foci are not associated with chromosome clusters, (ii) in the presence of microtubules, oocyte chromosome clusters are able to recruit the PCM which is necessary for spindle formation.

Table 2. *Effect of 10 μ M-nocodazole for 6 h on distribution of microvilli in unfertilized oocytes as judged by scanning electron microscopy*

Patterns of microvilli distribution	Control cells % (n = 43)	Nocodazole-treated cells % (n = 65)
Uniform microvillous	26	20
Microvillous free patches*		
1 small patch	51	25
1 large patch	23	23
2 patches		21
3 patches		3
No microvilli detected		8

* Note that the number of patches will underestimate the number of separate chromosome clusters since (a) the influence of chromatin on the cytocortex is quite extensive leading to coalescence of adjacent microvillous-free areas, and (b) it is not possible to examine the whole of the oocyte surface under the SEM.

Table 3. *Multiple polar body formation in eggs treated with 10 μ M-nocodazole after removal of the drug. Analysis was carried out after 8 h culture at 37°C*

Period of presence of the drug	Number of cells examined	% of eggs with given number of:											
		Chromosome clusters				Spindles or polar bodies*				Pronuclei (including male)†			
		1	2	3	>3	1	2	3	>3	1	2	3	>3
Control	95	100				100				4	96		
0-2 h	31	13	29	29	29	35	49	10		13	39	25	20
0-4 h	32	5		5	90	5	31	37	17				11
0-6 h	23	4	14	11	71	14	15	14	5				
0-8 h	59	4	18	13	65								

* Groups do not add up to 100 % because of the absence of formation of polar bodies in some eggs.

† Groups do not add up to 100 % because of the absence of formation of pronuclei in some eggs. When eggs are kept in nocodazole for the 8 h period, pronuclei do not form.

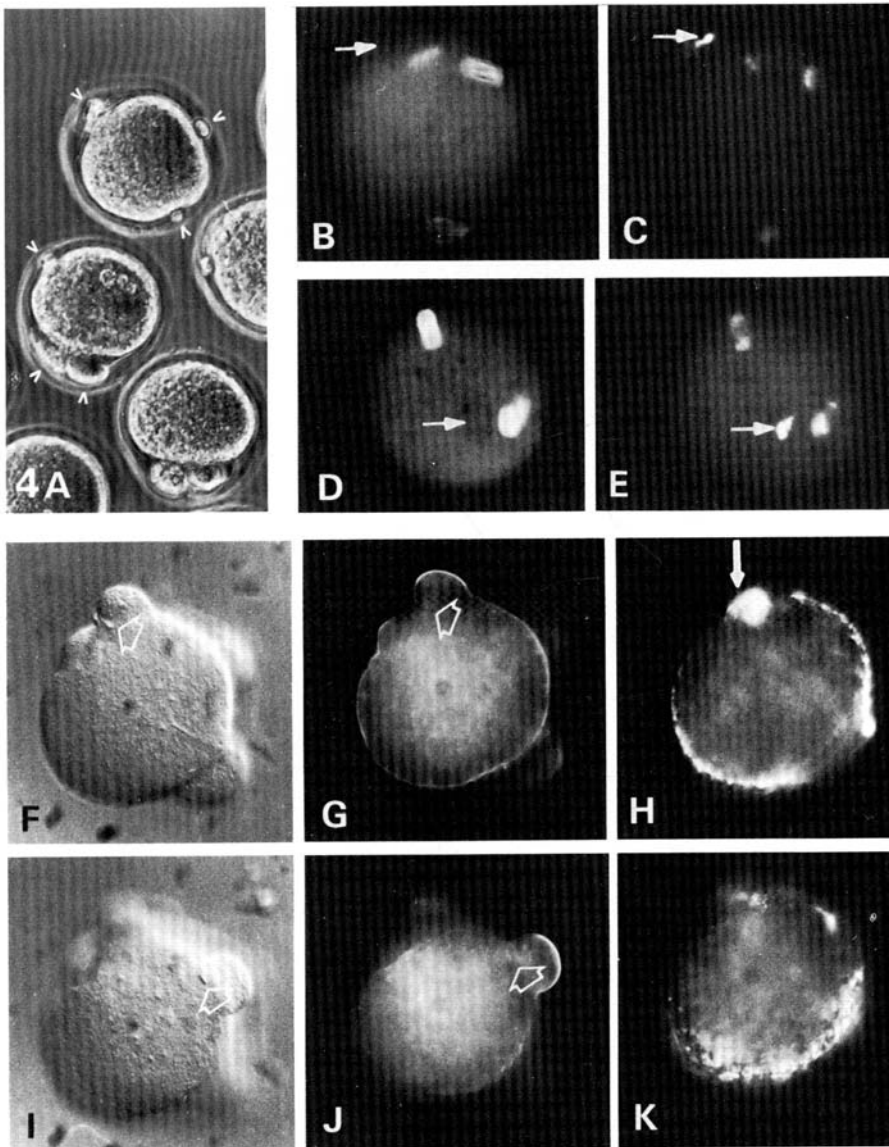


Fig. 4. Eggs treated with $10\text{ }\mu\text{M}$ -nocodazole for 1 h prior to insemination and for 4 h postinsemination, washed, and cultured in control medium for a further 4 h.

(A) Multiple polar body extrusion in living eggs. Arrowheads point at polar bodies.

(B,C) Egg double stained for tubulin (B) and chromatin (C). Note three metaphase spindles. The sperm-derived chromosomes (arrow) are not associated with a spindle.

(D,E) Egg double stained for tubulin (D) and chromatin (E). Note three rotating anaphase spindles. Arrows point to the sperm head which is not associated with a spindle.

(F-K) Egg double stained for actin (G,J) and Con A (H,K) and photographed at two different focal planes (F-H and I-K). Note two rotated spindles and polar bodies forming (open arrows). Another forming polar body can be seen out of focus (lower right). Large arrow points at the first polar body heavily stained with Con A. $\times 225$ (A), $\times 320$ (B-K).

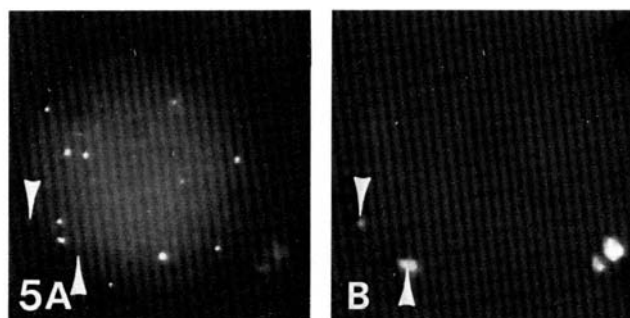


Fig. 5. (A,B) Oocyte treated with $10\text{ }\mu\text{M}$ -nocodazole for 6 h double stained for PCM (A) and chromatin (B). Note that PCM foci are not associated with the two chromosome clusters (arrowheads) which can be observed in this plane of focus (first polar body stains for chromatin only, lower right). $\times 400$.

Chromosomes and microtubules

It has been shown that in oocytes arrested at metaphase II of meiosis, the dispersed cytoplasmic PCM foci do not nucleate microtubules, the only microtubules found being those forming the spindle (Wassarman & Fujiwara, 1978; Maro *et al.* 1985; Schatten *et al.* 1985; Fig. 1A). In order to test whether the oocyte chromosomes themselves promote tubulin polymerization in their vicinity

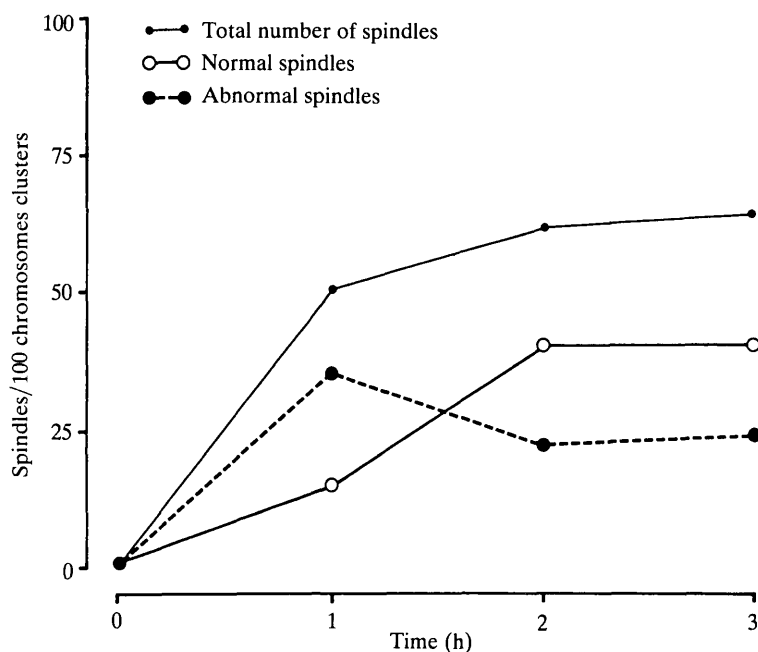


Fig. 6. Spindle reformation in nocodazole-treated oocytes ($10\text{ }\mu\text{M}$ for 4 h) cultured for various periods of time in control medium after removal of the drug. In this experiment a spindle is defined as a chromosome cluster located on a microtubule bundle. Spindles are categorized as normal if PCM can be detected unambiguously at both poles. Abnormal spindles are those in which we could not identify a PCM focus at both poles. 21 cells were examined at 1 h, 122 at 2 h and 34 at 3 h.

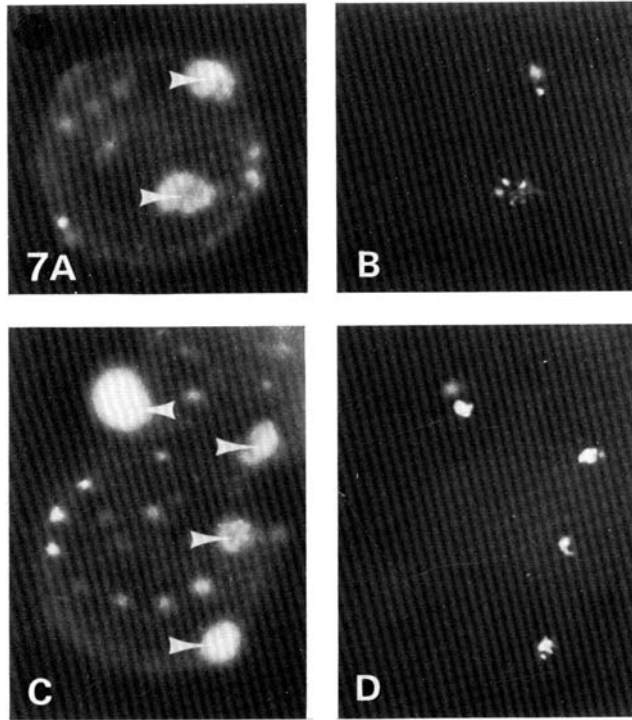
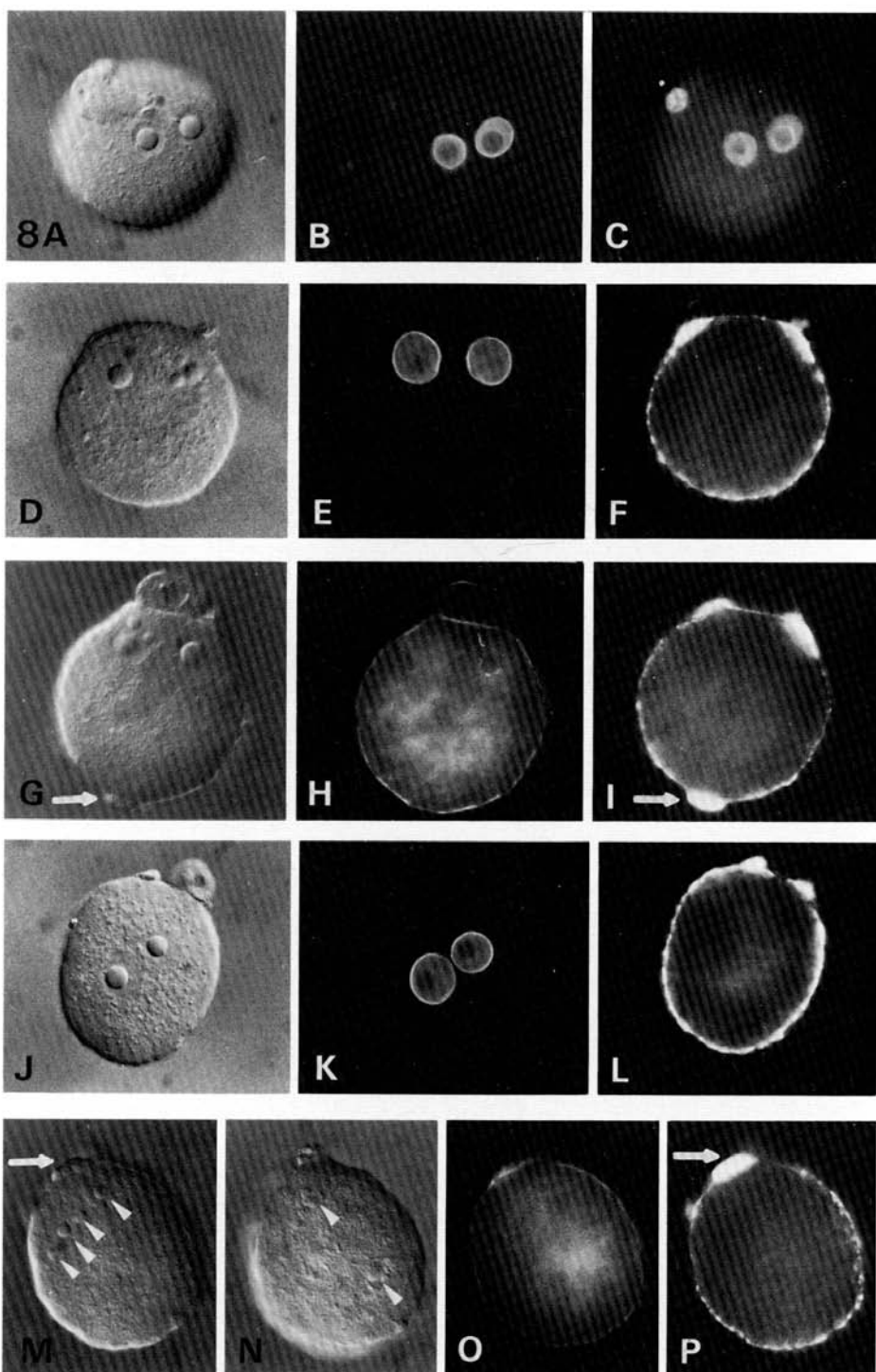


Fig. 7. (A–D) Oocytes treated with $10\mu\text{M}$ -nocodazole for 6 h, washed and immediately incubated in $6\mu\text{M}$ -taxol for 5 min at 37°C , fixed and double stained for tubulin (A,C) and chromatin (B,D). Note microtubule bundles associated with chromosome clusters (arrowheads) and the small asters nucleated by PCM foci. $\times 400$.

as has been shown in frog oocytes (Karsenti, Newport, Hubble & Kirschner, 1984), we used taxol to decrease the critical concentration for tubulin polymerization (Schiff, Fant & Horwitz, 1979). Unfertilized oocytes, in which chromosomes and PCM had been dispersed by nocodazole, were incubated in the presence of taxol for 5 min and then immediately fixed so that no association between PCM foci and chromosomes could form and stained with an anti-tubulin antibody. Large bundles of microtubules were observed around most of the chromosome clusters (380 out of 460; 83%; Fig. 7), whilst the separate small asters were nucleated by the cytoplasmic PCM foci (Maro *et al.* 1985). From this result we conclude that chromosome clusters favour microtubule polymerization in their vicinity.

Formation and migration of pronuclei

In no egg treated continuously with nocodazole did pronuclei form, as assessed both by differential interference contrast microscopy and staining with antibodies to nuclear lamins (Table 4; Fig. 8A–F). Moreover, in nocodazole-treated eggs, central migration and aggregation of sperm- and oocyte-derived chromosome clusters failed to occur. When nocodazole was removed 2 h after insemination,



multiple pronuclei formed in many eggs, each corresponding to a dispersed chromosome cluster, but no migration of these pronuclei occurred (Table 4) even when eggs were incubated for 12 h, well beyond the normal time of migration. These results are consistent with the observations that *in vivo* colchicine induces an increase in egg ploidy (Edwards, 1958). When nocodazole was removed at times later than 4 h, pronuclear formation did not occur at 8 h (Table 4). Conversely, if eggs were placed in nocodazole at 2 h or later postinsemination, the incidence of pronuclear formation was unaffected (Table 4; Fig. 8D–L) but migration was suppressed completely (Table 4; Fig. 8D–I). The drug did not reverse migration when it had already occurred (Table 4; Fig. 8J–L).

Having established the conditions required to disperse chromosomes and to control both their incorporation into pronuclei and their centrally directed migration, we examined next the cytocortical changes under these various conditions. When nocodazole was washed out at 2 h, pronuclei formed and cortical changes were lost (Table 4; Fig. 8M–P). This loss occurred despite the failure of pronuclei to migrate centrally. In contrast when removal of nocodazole was delayed beyond 2 h pronuclei did not form and the cytocortical changes adjacent to the chromosomes persisted (Fig. 4F–K; Table 4). These results suggested that only chromosomes not located in a nucleus could induce the focal response. Confirmation of this impression came from the analysis of eggs that were first transferred to nocodazole at 2 h postinsemination or later. In most of these eggs pronuclei formed but did not migrate, and in most, foci of reduced Con A binding and elevated focal actin were also absent. Indeed, where migration had not

Fig. 8. (A–C) Fertilized egg 6 h postinsemination stained for nuclear lamins (B,C). Note that a very weak lamin staining is seen around the chromosomes extruded in the second polar body (C; as visualized by overexposure of Fig. 6B) probably corresponding to a reduced quantity of available lamin molecules in the polar body rather than to a selective mechanism to avoid normal nuclear envelope formation in the polar body.

(D–F) Egg transferred to medium containing 10 μ M-nocodazole 2 h postinsemination and cultured for a further 6 h before staining for nuclear lamins (E) and Con A (F). Note that pronuclei form but do not migrate towards the centre of the egg and that the membrane overlying the pronuclei is enriched in Con A binding sites.

(G–I) Egg transferred to medium containing 10 μ M-nocodazole 4 h postinsemination and cultured for a further 4 h before staining for actin (H) and Con A (I). Note non-migrated pronuclei with enriched Con A binding in the adjacent plasma membrane (I) whole cortical actin is evenly distributed (H). Arrow points at the dead first polar body which stains heavily with Con A.

(J–L) Egg transferred to medium containing 10 μ M-nocodazole 6 h postinsemination and cultured for a further 2 h before staining for nuclear lamins (K) and Con A (L). Note that pronuclei form and migrate towards the centre of the egg and that Con A binds evenly to the cell surface.

(M–P) Egg cultured in medium containing 10 μ M-nocodazole for 1 h prior to insemination and for 2 h postinsemination, at which time nocodazole was removed and the egg cultured for a further 6 h in control medium, stained for actin (O) and Con A (P). Note multiple non-migrated pronuclei (six are visible in M and N) with an even distribution of cortical actin (O) and Con A binding (P). Arrow points at the dead first polar body which stains heavily with Con A. $\times 320$.

Table 4. *Effect of pronuclear formation and migration on cortical changes in fertilized eggs*

Incubation in presence of:				% of eggs with pronuclei:		% of eggs where chromosomes are associated with an area:		
Control medium	Nocodazole 10 μ M	Control medium	Cell number	Formed	Migrated	Rich in actin	Con A negative	Con A positive
0-2 h			10			100	100	
0-4 h			32	82		56	78	
0-6 h			27	100	40	11	18	
0-8 h			95	100	77		2	
	0-2 h	2-8 h	31	97		16	16	
	0-4 h	4-8 h	32	11		91	91	
	0-6 h	6-8 h	27			100	100	
	0-8 h		59			100	97	
0-2 h	2-8 h		45	100			11	82
0-4 h	4-8 h		60	100			16	65
0-6 h	6-8 h		66	100	32		10	47

occurred, an elevated Con A binding was associated with the underlying pronuclei in most cases (Table 4; Fig. 8D-I). This elevated binding was not accompanied by any obvious inequity in the subcortical distribution of actin (Fig. 8G-I).

Eggs in which elevated Con A binding had occurred were also examined using scanning and transmission electron microscopy. One or two areas of blebby microvilli were observed in most of these eggs under the scanning electron microscope (Fig. 9A). This result was confirmed by observation of thin sections under the transmission electron microscope where large microvilli and blebs were observed in the portion of the cell cortex overlying the pronuclei (Fig. 9B,C).

From these results we conclude that (i) only chromosomes not located in a nucleus are able to induce the cortical changes, (ii) the reestablishment of a normal microvillous cortex over the pronuclei is sensitive to nocodazole and may therefore require microtubules, (iii) microtubules are required for the migration of pronuclei, (iv) nocodazole prevents the appearance of pronuclei possibly by a direct effect, but more probably indirectly (see Discussion).

DISCUSSION

In this paper we have demonstrated three types of interaction between the chromosomes and the cytoskeleton. First, chromosomes which are not contained in a nucleus are associated with an extensive subcortical focus of microfilaments and the loss of overlying microvilli. Second, condensed meiotic chromosomes promote microtubule growth in their vicinity. Third, when condensed meiotic chromosomes surrounded by microtubules associate with pericentriolar material, a spindle forms. Together, these three interactions go some way towards explaining the mechanism of polar body formation.

Previous work has shown that two domains exist in the egg cortex, a small domain covering the spindle area rich in microfilaments and devoid of microvilli

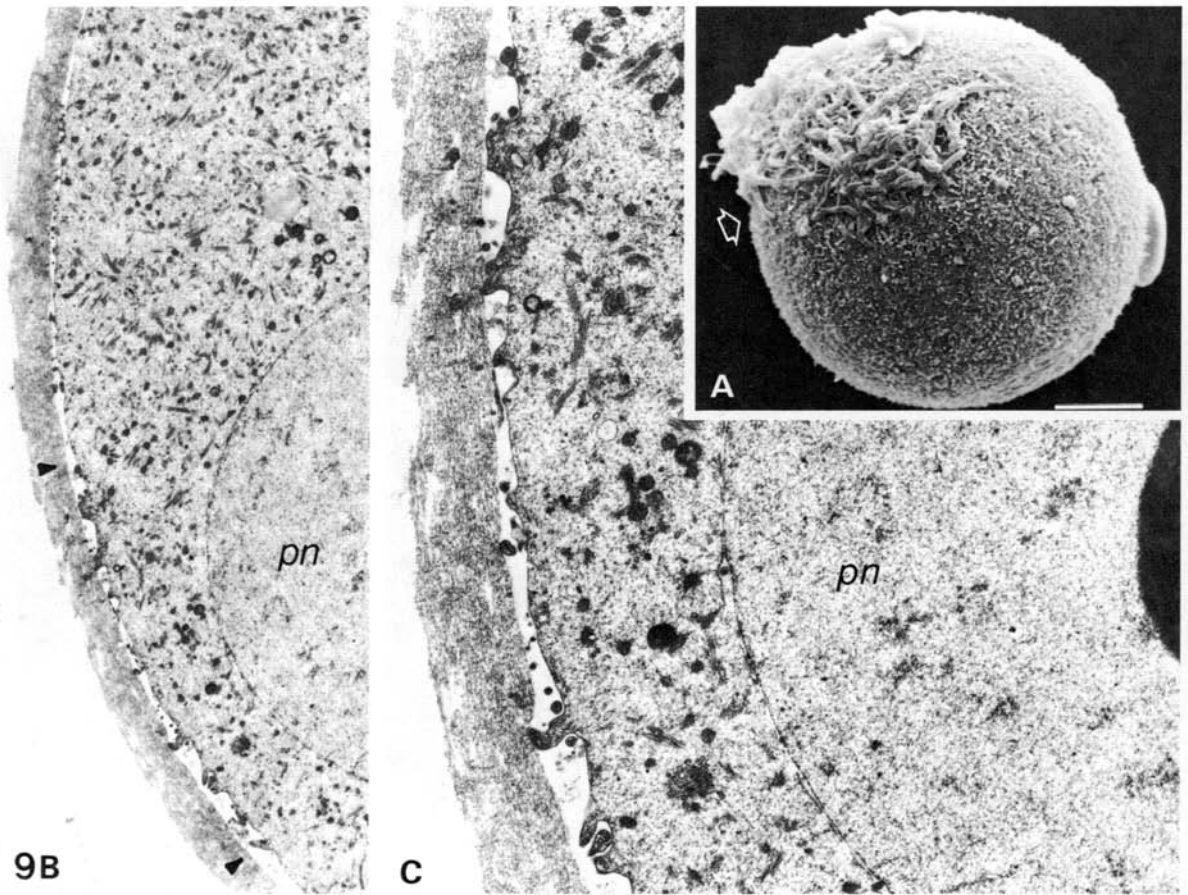


Fig. 9. (A) Scanning electron microscopy of an egg which was transferred to medium containing $10\text{ }\mu\text{M}$ -nocodazole 2 h postinsemination and cultured for a further 6 h. Note protrusion of membranes and microvilli (open arrow). $\times 1260$.

(B,C) Transmission electron microscopy of an egg which was transferred to medium containing $10\text{ }\mu\text{M}$ -nocodazole 2 h postinsemination and cultured for a further 6 h. Note protrusion of membranes and microvilli in the portion of the plasma membrane facing the pronucleus (defined by arrowheads in B); C is an enlargement of this area and reveals no obvious structural links between the pronucleus and the overlying membrane. *pn*, pronucleus. $\times 4725$ (B), $\times 10800$ (C).

Note that for SEM it is essential to remove the zonae pellucidae and thus the membrane protrusions appear more marked than when compressed under the zona and viewed only in thin section.

and a large one covering the rest of the cell surface rich in microvilli and containing less microfilaments (Eager *et al.* 1976; Johnson *et al.* 1975; Maro *et al.* 1984; Longo & Chen, 1985; Karasiewicz & Soltynska, 1985; Van Blerkom & Bell, 1986). Spermatozoa do not usually fertilize the egg in the area devoid of microvilli (Johnson *et al.* 1975). When the sperm head decondenses with loss of its nuclear membrane (Stefanini, Oura & Zamboni, 1969) a cortical domain similar to that existing near the meiotic chromosomes is induced (Shalgi *et al.* 1978; Maro *et al.* 1984). This latter observation added to the fact that after nocodazole-induced

dispersion of the oocyte chromosomes, each cluster of chromosomes is able to induce the formation of one of these domains despite the absence of associated microtubules (see also Longo & Chen, 1985), suggested that this cortical domain was induced by chromatin rather than by spindle microtubules. Similarly, although links between MTOCs and the nuclear envelope have been documented in many systems (Bornens, 1977; Maro & Bornens, 1980; Kallenback & Mazia, 1982; Tassin, Maro & Bornens, 1985), including mouse oocytes (Calarco, Donahue & Szollosi, 1972; Maro *et al.* 1985) we were unable to detect any role for them in mediating chromosome-induced changes in the cell cortex, since an antigen linked to the pericentriolar material (Calarco-Gillam *et al.* 1983; Maro *et al.* 1985) was not associated with the chromosomes in nocodazole-treated oocytes. Only 1–3 h after removal of nocodazole did pericentriolar material and chromosomes come together to form functional spindles.

This effect of chromatin on the cell cortex explains the existence of the fertilization cone which develops after sperm entry into the egg. The sperm nuclear envelope breaks down because of the meiotic environment persisting in the oocyte during the first 30 min after fertilization, the metaphase–anaphase transition occurring only 20 to 30 min after the fusion between the oocyte and the sperm head (Sato & Blandau, 1979). The presence of the non-enveloped male chromatin then induces a changed cortical domain. In addition, previous observations suggested that the cleavage furrow of meiosis II (and thus polar body formation) could only take place in one of these domains, rich in microfilaments and devoid of microvilli (Eager *et al.* 1976; Johnson *et al.* 1975; Maro *et al.* 1984). Our present work has confirmed these previous suggestions: when chromosomes are dispersed by nocodazole prior to fertilization and when the drug is removed later, multiple polar bodies form in many of the cortical domains associated with the chromosomes. We do not at present understand what, if anything, controls the direction of rotation of the spindle, and thereby determines which haploid set of chromosomes are discarded in the polar body.

However, polar body formation requires not merely a modified cortical domain, but also an organized spindle. The oocyte chromatin, but not sperm chromatin, has two properties that allow elaboration of spindles. First, we have shown that condensed chromosomes promote tubulin polymerization, thereby allowing microtubule formation. Thus, most of the chromosome clusters observed after nocodazole-induced dispersion are not linked to associated MTOCs (as defined by the anti-PCM serum) but nonetheless promote the formation of large microtubule bundles after taxol treatment (this may be more readily apparent because of the increased concentration of free tubulin which has been induced by nocodazole prior to taxol addition). Moreover, we know that inactive PCM foci exist in the cytoplasm of the normal metaphase II arrested oocyte, but they do not nucleate microtubules unless the critical concentration for tubulin polymerization is reduced experimentally (Maro *et al.* 1985), whereas microtubules are clearly present in the vicinity of the chromosomes, again suggesting that chromatin promotes polymerization. Second, we have shown here that in the presence of microtubules,

oocyte meiotic chromosomes can recruit PCM foci and, in conjunction with the promoting effect of meiotic chromosomes on tubulin polymerization, a spindle is organized.

We do not know how chromosomes influence either tubulin or actin polymerization in their vicinity. The effects of chromosomes on actin and the cell cortex seem to be unique to chromosomes in the meiotic oocyte and newly fertilized interphase mouse egg, as we have not observed such an effect in other mouse cells. However an analogous situation has been described in the *Drosophila* blastoderm (Warn, Magrath & Webb, 1984). It is of interest that a feature common to both these systems is the peripheral location of the chromosomes, and this may prove to be the most important determinant of whether the effect is observed. In contrast, the effects on tubulin polymerization and MTOC recruitment appear to be a general property of chromosomes in the meiotic or mitotic state. Thus, a decrease in the apparent critical concentration for tubulin polymerization in the vicinity of the chromosomes has also been observed in *Xenopus* oocytes (Karsenti *et al.* 1984). In this latter case, centrosomes injected into the cytoplasm of a metaphase arrested egg were not able to nucleate microtubules unless located close to the chromosomes. However, following activation at fertilization in both *Xenopus* and the mouse, all of the MTOCs dispersed throughout the egg were able to nucleate, suggesting that a drop in the cytoplasmic critical concentration for tubulin polymerization occurs during interphase (Karsenti *et al.* 1984; Maro *et al.* 1985; Schatten *et al.* 1985). Moreover, a dependence of the total length of spindle microtubules on the number of chromosomes present in the spindle has been demonstrated in grasshopper spermatocytes (Nicklas & Gordon, 1985).

The effect on tubulin polymerization of the transition from meiosis or mitosis to interphase may reflect a fall in the level of maturation promoting factor (MPF), the activity of which oscillates during the cell cycle (Masui & Markert, 1971; Wasserman & Smith, 1978; Gerhart, Wu & Kirschner, 1984). In the egg, MPF is stabilized prior to fertilization by the activity of cytostatic factor (CSF). CSF is found only in unfertilized eggs and has been shown to cause metaphase arrest upon injection into cleaving eggs (Masui, Meyerhof & Miller, 1980). MPF induces nuclear membrane breakdown and chromosome condensation when injected into eggs arrested at the end of S phase (Miake-Lye, Newport & Kirschner, 1983), hence loss of the sperm nuclear membrane after fertilization. This factor might also regulate, directly or indirectly, microtubule assembly into spindles in mitotic cells. Both CSF and MPF activities fall rapidly after fertilization as evidenced by the incapacity of the egg to condense chromosomes or break down nuclear membranes within 30–60 min after activation (Czołowska, Modlinski & Tarkowski, 1984; Howlett & Maro, unpublished observation) and there is evidence that their loss is dependent on a transient Ca^{2+} influx at fertilization that destroys CSF and destabilizes MPF (Newport & Kirschner, 1984). However, it is clear from our experiments here that when fertilization occurs in the presence of nocodazole, MPF activity (and perhaps CSF activity) must persist for at least 4 to 6 h. Thus

removal of nocodazole at any point during this period is associated with rapid spindle formation in the vicinity of the condensed chromosomes. Since there is evidence that MPF drives microtubular and nuclear membrane changes during the cell cycle, rather than being dependent upon them (Miake-Lye *et al.* 1983), it seems likely that the maintained level of MPF (and perhaps CSF) in the presence of nocodazole may be secondary to an effect of the drug on the normal mechanism by which these activities are destroyed or maintained.

Although the precise mechanisms by which the chromosomes are able to induce changes in the organization of both microfilaments and microtubules are unclear, it is nevertheless possible to propose a sequence for the various events leading to polar body formation. First it seems that at the end of maturation, just before germinal vesicle break down, the chromosomes gain the capacity to modify the organization of actin in their vicinity (Van Blerkom & Bell, 1986). Second, when the germinal vesicle breaks down at entry into metaphase, the MTOC material (PCM) which was associated previously with the nuclear envelope, becomes dispersed in several foci within the cytoplasm (Calarco *et al.* 1972; Maro *et al.* 1985). At the same time a spindle is formed because of the effect of the chromosomes on tubulin polymerization during M-phase (Karsenti *et al.* 1984; our data) in conjunction with the recruitment of nearby MTOCs. Third, the spindle moves towards the periphery of the cell in a microfilament-dependent process (Longo & Chen, 1985), probably related to the effect of chromatin on the microfilament network (Van Blerkom & Bell, 1986). Finally, when the spindle reaches the cell periphery, the chromosomes induce the formation of an actin-rich, microvillous-free domain of the cortex (Maro *et al.* 1984; Longo & Chen, 1985; Van Blerkom & Bell, 1986). After fertilization (or activation), meiosis resumes and, after the metaphase–anaphase transition, the cleavage furrow forms only in this microfilament-rich domain of the cortex (Maro *et al.* 1984). The rotation of the spindle follows (possibly as a consequence of the limited area available for the development of the furrow), the cleavage furrow is completed, and the polar body forms (Maro *et al.* 1984). Having defined the various steps of the sequence, and some of the underlying mechanisms, it remains to determine the nature of their molecular interrelationships in more detail.

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