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Activation of *in vitro* matured mouse oocytes arrested at first or second meiotic metaphase

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ABSTRACT Some mammalian oocytes fail to complete maturation *in vitro* and arrest development at the first metaphase stage. The response of such blocked oocytes to sperm penetration was investigated. Ovarian mouse oocytes from two inbred strains, CBA/Kw and KE, were cultured *in vitro* for 20 h. Both oocytes arrested at the first metaphase (MI oocytes) and second metaphase (MII oocytes) were then inseminated. The majority of MII and MI oocytes reinitiated meiosis in response to sperm penetration, although those from the CBA strain did with higher frequency. Moreover, a high proportion of unpenetrated oocytes from CBA, but not the KE strain, resumed meiosis (33% for MII and 48% for MI oocytes, respectively). Parthenogenetic activation of MI-arrested oocytes was demonstrated in (CBAxKE)F_1 mice; ovarian oocytes matured *in vitro* and then treated by electric shock were activated with a similar total frequency of 52.4% for MI and 47.8% for MII oocytes. The rate of activation increased equivalently for both MI and MII oocytes as the length of maturation prolonged. This demonstrates that mouse oocytes arrested at MI during their maturation *in vitro* continue cytoplasmic maturation and become capable of undergoing activation in a way similar to those maturing to MII. Additionally, in MII oocytes cultured for an equal time *in vitro* the rate of activation increased with the time lapse after first polar body (PB1) extrusion. This indicates that after PB1 extrusion, the oocyte requires some resting time before it may be activated, perhaps to restore the proper balance between elements of the cell cycle controlling the mechanism involved in first meiotic division.

KEY WORDS: *mouse oocyte, meiotic maturation, metaphase arrest, activation, in vitro*

Introduction

In mammals, when oocytes are released from antral follicles into culture medium and allowed to undergo spontaneous maturation *in vitro*, some fail to complete maturation. In such oocytes, rather than extruding the first polar body (PB1) and completing maturation to the second meiotic metaphase (MII), the progression of meiosis is arrested at the first (MI) metaphase (Bae and Foote, 1980; Tsuji *et al.*, 1985; Motlik and Fulka, 1986; Polanski, 1986; Bagger *et al.*, 1987; Didion *et al.*, 1990; Meinecke and Meinecke-Tillmann, 1993). The proportion of MI-arrested oocytes may vary depending on different experimental conditions, for example, donor species (Motlik and Fulka, 1986; Didion *et al.*, 1990), follicle or oocyte diameter (Sorensen and Wassarman, 1976; Tsuji *et al.*, 1985; Motlik and Fulka, 1986), phase of the follicular cycle (Tsuji *et al.*, 1985) and the composition of the medium used for oocyte isolation (Bagger *et al.*, 1987) or culture (Bae and Foote, 1980). It has been recently reported that bovine oocytes arrested in MI during their maturation *in vitro* completed maturation when fertilized (Chian *et al.*, 1992).

The aim of this study was to examine in what way mouse oocytes blocked at MI during maturation *in vitro* respond to sperm penetration. Two inbred strains of mice, CBA/Kw (henceforth called CBA) and KE, were used. These strains differ greatly in some oocyte characters as, for example, efficiency of fertilization *in vivo* (Krzanowska, 1970) and *in vitro* (Kaleta, 1977), or the number of cortical granules (Wabik-Sliz, 1979). Oocytes of these strains differ also in the speed of meiotic maturation measured both as the time of PB1 extrusion (Polanski, 1986) and as the time of acquisition of the capacity to transform sperm heads into pronuclei (Polanski, 1990). The use of such different types of mouse oocytes allowed us to study possible interstrain differences in oocyte behavior.

Results

During this study ovarian oocytes from CBA and KE mice were cultured *in vitro* to complete maturation. In both strains a

Abbreviations used in this paper: MI, first metaphase; MII, second metaphase; GV, germinal vesicle; PB1, first polar body.

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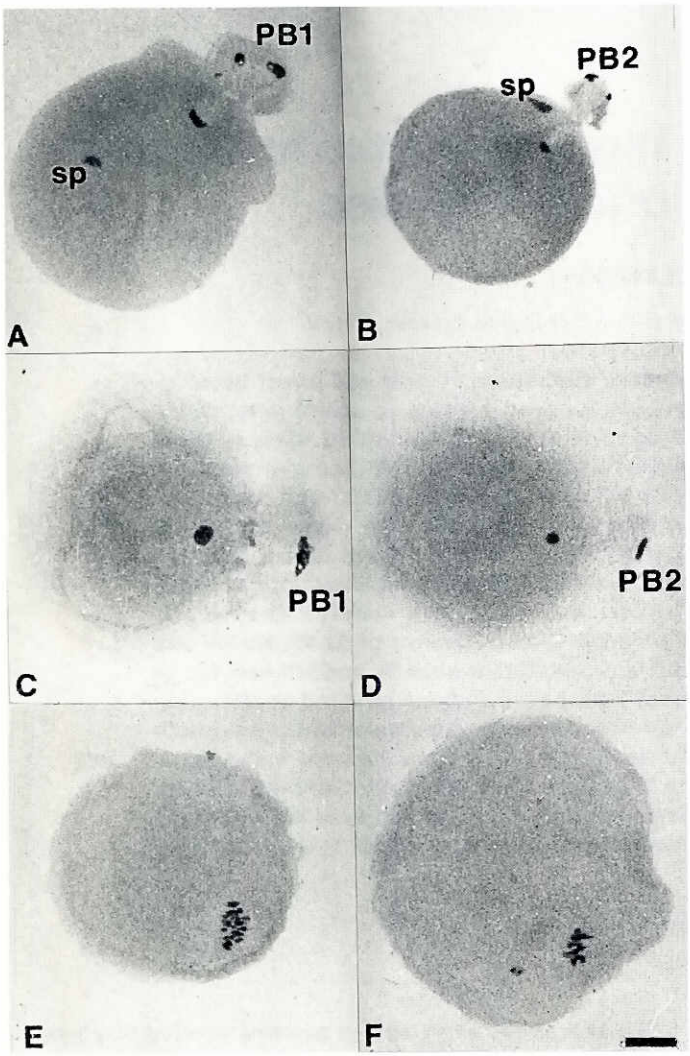

Fig. 1. Behavior of *in vitro* matured oocytes after insemination. *Air-dried, toluidine blue stained preparations showing:* **(A)** *monospermic KE oocyte leaving MI arrest (first telophase);* **(B)** *monospermic CBA oocyte in second meiotic telophase;* **(C)** *unpenetrated CBA oocyte reinitiating first meiosis (telophase);* **(D)** *unpenetrated CBA oocyte in second meiotic telophase (PB1 was removed before insemination);* **(E,F)** *control non-inseminated KE oocytes with intact first* **(E)** *and second* **(F)** *metaphase plates. sp, sperm; PB1, first polar body; PB2, second polar body. Differences in oocyte size result from different degrees of oocyte flattening on the slide. Bar, 20 μM.*

small proportion of oocytes was unable to resume meiosis and after 20 h of culture were found to be still at the GV stage. Among those oocytes which resumed maturation during 20 h of culture, most extruded PB1 but some (about 30% in each strain) failed to do so. These two types of oocytes were considered as arrested at MII and MI, respectively.

In both strains, following insemination, significantly more MII oocytes were penetrated by spermatozoa than MI oocytes (Fig. 1A,B). Unexpectedly, the mean number of penetrating spermatozoa was higher in penetrated MI oocytes, and in the CBA strain this difference was statistically significant (Table 1).

The majority of penetrated oocytes, both MI and MII, reinitiated meiosis (Table 2). Such oocytes were found in anaphase or, more often, in telophase stage (first or second, depending on the type of inseminated oocyte; Fig. 1A,B). The proportion of penetrated oocytes resuming meiosis was higher in CBA strain, where 90.9% and 83.6% of inseminated oocytes left MI or MII arrest, respectively. These values in KE strain were 65% and 67.6%, respectively. Control non-inseminated oocytes remained (with one exception in KE strain) undisturbed in MI or MII stage (Fig. 1E,F). Moreover, in the CBA strain a relatively high proportion of both MI and MII inseminated oocytes reinitiated meiosis in the absence of sperm penetration (Fig. 1C,D), which was not observed in non-inseminated control oocytes from this strain (Table 2). Similar activating ability of sperm suspension without sperm penetration was previously described by Kaufman (1973).

Table 2 shows that in all groups oocytes from the CBA strain resumed meiosis with a higher frequency than oocytes from the KE strain. When the data for MI and MII oocytes were pooled (oocytes in both metaphases behave similarly) the strain difference was clearly significant both for penetrated as well as unpenetrated oocytes (Table 2).

The ability of MI-arrested oocytes to undergo parthenogenetic activation was demonstrated by applying a commonly used activating agent. Table 3 shows the efficiency of electric field mediated activation of oocytes from F_1 mice (CBAXKE) matured *in vitro* for 14, 16 or 18 h. Two types of activation were observed: full activation (oocytes which developed pronuclei) and abortive activation characterized by the return of the oocyte to a metaphase state after polar body extrusion (Kubiak, 1989). A few of the control untreated MI oocytes extruded PB1 and progressed to the MII stage as a result of delayed maturation. This probably occurred also in the electric field-treated group of MI oocytes and should be taken into account. For this reason, the number of abortively activated MI oocytes in treated groups was corrected by subtracting the putative number of maturing oocytes (calculated from appropriate control groups) from the total number of oocytes found in metaphase and with extruded PB1. Thus, in Table 3 in the column referring to abortive activation of MI oocytes two values are given for each treated group, the first (no parentheses) being the corrected one. The last col-

TABLE 1

SPERMATOZOA PENETRATION OF OOCYTES FROM CBA AND KE STRAIN INSEMINATED *IN VITRO* AFTER MATURATION IN CULTURE FOR 20 H

Strain	oocyte status at insemination	No. of oocytes	No. (%) of penetrated oocytes	Mean no. of sperm±SEM in penetrated oocytes
CBA	MI	32	11 (34.4)[a]	2.40±0.56*
	MII	79	61 (77.2)[a]	1.56±0.10*
KE	MI	39	20 (51.3)[b]	2.00±0.23
	MII	80	71 (88.8)[b]	1.90±0.16

Data pooled from 12 independent experiments; 3 involving only MII and 9 involving both MI and MII oocytes. Values with identical superscript differ significantly: [a,b]$P<0.0001$ (χ^2 test of independence); *$P<0.02$ (ANOVA).

TABLE 2

BEHAVIOR OF *IN VITRO* MATURED OOCYTES FROM INBRED MOUSE STRAINS CBA AND KE AFTER INSEMINATION

Strain	oocyte status at insemination	sperm penetration status	No. of oocytes	No.(%) of oocytes that resumed meiosis
CBA	MI	penetrated	11	10 (90.9)[a]
		unpenetrated	21	10 (47.6)[b]
		control	20	0[a,b]
	MII	penetrated	61	51 (83.6)[c]
		unpenetrated	18	6 (33.3)[d]
		control	24	0[c,d]
KE	MI	penetrated	20	13 (65.0)[e]
		unpenetrated	19	1 (5.3)
		control	25	1 (4.0)[e]
	MII	penetrated	71	48 (67.6)[f]
		unpenetrated	9	1 (11.1)
		control	18	0[f]
CBA	MI, MII	penetrated	72	61 (84.6)*
KE	MI, MII	penetrated	91	61 (67.0)*
CBA	MI, MII	unpenetrated	39	16 (41.0)**
KE	MI, MII	unpenetrated	28	2 (7.1)**

Data pooled from 12 independent experiments; 3 involving only MII and 9 involving both MI and MII oocytes. Values with identical superscripts differ significantly (χ^2 test of independence). Differences between control and inseminated oocytes within experimental groups: [a,c,e,f]$P<0.0001$; [b]$P<0.002$; [d]$P<0.01$. Differences between strains: *$P<0.02$; **$P<0.01$.

umn in Table 3 gives only the corrected total numbers of activated oocytes. As shown in Table 3, MI and MII oocytes activated with a similar frequency; the total number of activated MI and MII oocytes were 52.4% and 47.8%, respectively. For both MI and MII oocytes, the ability to undergo activation increased as the period in culture was prolonged from 14 to 18 h. However, whereas abortive activation was rare (2/22) in MI oocytes, it occurred in more than half (29/54) of the MII oocytes. The difference in the pattern of activation with respect to polar body formation was also noted. Among 22 activated MI oocytes, 9 (40.9%) extruded the first polar body, 12 (54.5%) underwent immediate cleavage and 1 (4.5%) formed a single pronucleus without cell division. In the MII group 39 oocytes (72.2%) formed a second polar body, 4 (7.4%) underwent immediate cleavage and 11 (20.4%) remained undivided developing one (7 oocytes) or two (4 oocytes) pronuclei.

As shown in Table 4, the ability of MII oocytes to undergo activation increased gradually following PB1 extrusion. Oocytes treated with electric pulses 2-4 h after PB1 extrusion did not become activated at all, whereas those treated 4-6, 6-8 and 8-10 h after PB1 extrusion were activated with a frequency of 34.4%, 59% and 74.1%, respectively.

Some of the oocytes from F_1 mice which failed to extrude PB1 during maturation *in vitro* were analyzed for their chromosome configuration, instead of being electro-stimulated. This analysis was undertaken to confirm their nuclear state, because it was possible that they had progressed to MII without extrusion of PB1. Of 24 putative MI oocytes examined in this way after 18-24 h of culture, 20 (83.3%) contained a chromosome configuration typical of MI (Fig. 2A), with only 4 (16.7%) containing an MII configuration (Fig. 2B).

Some F_1 oocytes activated fully after electric field treatment were cultured overnight in the presence of colcemide (1 μg/ml). Next day, after the disappearance of the pronuclei, they were fixed for chromosome analysis. Figure 2C and D shows a haploid and a diploid mitotic metaphase plate from an activated MII and MI oocyte, respectively. This shows that oocytes activated at MI are able to progress through the first mitotic cycle, similar to MII oocytes.

Discussion

As expected (see Introduction) not all of the oocytes from both KE and CBA strains which resumed maturation *in vitro* completed it. A number of oocytes were found arrested in MI after 20 h of culture. After insemination, significantly more MII oocytes from both strains had undergone penetration than MI oocytes (Table 2). This might suggest that MI arrest may be associated with a reduced ability of the oocyte plasma membrane to fuse with a spermatozoon, which is necessary for subsequent sperm entry into cytoplasm (Van Blerkom and Motta, 1979). However, it is difficult to explain why, when sperm entry occurred, MI oocytes were penetrated with a similar (in KE strain), and significantly higher (in CBA strain) number of spermatozoa than MII oocytes.

The results presented here confirm those obtained for bovine oocytes (Chian *et al.*, 1992), whereby oocytes arrested at the MI stage during *in vitro* maturation may be stimulated to reinitiate

TABLE 3

ACTIVATION OF OOCYTES FROM F_1 MICE AFTER DIFFERENT PERIODS OF MATURATION *IN VITRO*

oocyte status	duration of culture (h)	treatment	No. of oocytes	No. of activated oocytes: abortive	full	total (%)
MI	14	-	14	3*	0	-
	14	+	12	0 (3)**	1	1/9 (11.1)
	16	-	11	1*	0	-
	16	+	17	0 (2)**	8	8/15 (53.3)
	18	-	11	1*	0	-
	18	+	20	2 (2)**	11	13/18 (72.2)
MII	14	+	25	4	0	4/25 (16.0)
	16	+	42	13	8	21/42 (50.0)
	18	+	46	12	17	29/46 (63.0)
	18	-	26	0	0	0/26 (0.0)
MI	total 14-18	+	49	2 (7)**	20	22/42 (52.4)[a]
MII	total 14-18	+	113	29	25	54/113 (47.8)[a]

Data pooled from three independent experiments (4 females/exp.). *not activated but delayed extrusion of the first polar body during the course of maturation. **numbers in parentheses refer to the oocytes recognized as not activated but delayed in the maturation (see text in Results). [a]difference not statistically significant; $P>0.7$ (χ^2 test of independence).

TABLE 4

ACTIVATION OF *IN VITRO* MATURED MII OOCYTES FROM F_1 MICE IN RELATION TO THE TIME OF CULTURE AND THE TIMING OF PB1 EXTRUSION

Duration of maturation (h)	time after PB1 extrusion (h)	No. of oocytes	No. of activated oocytes		
			abortive	full	total (%)
14	2-4	11	0	0	0 (0.0)
	4-6	14	4	0	4 (28.6)
16	4-6	18	4	3	7 (38.8)
	6-8	24	9	5	14 (58.3)
18	6-8	15	2	7	9 (60.0)
	8-10	27	10	10	20 (74.1)
14-18	2-4	11	0	0	0 (0.0)
	4-6	32	8	3	11 (34.4)
	6-8	39	11	12	23 (59.0)
	8-10	27	10	10	20 (74.1)

Data pooled from three independent experiments (4 females/exp.).

meiosis by sperm penetration. Moreover, these data show that such oocytes are also able to undergo parthenogenetic activation and progress to the first mitotic metaphase (40 mitotic chromosomes, see Fig. 2D), similar to MII oocytes. In fact, first and second meiotic metaphases are very similar in their biochemical character – both are maintained by a high level of activity of maturation promoting factor (MPF), a universal regulator of cell cycle control (Murray, 1992). The extrusion of both the first and the second polar body is accompanied by sudden disappearance of this activity (Choi *et al.*, 1991; Kubiak *et al.*, 1992) resulting from the rapid destruction of cyclin B, the regulatory subunit of MPF (Murray *et al.*, 1989; Weber *et al.*, 1991; Hampl and Eppig, 1995). However, triggering the pathway leading to cyclin degradation at second meiotic division depends on external factors (sperm penetration or parthenogenetic stimulus), which activate the egg, whilst at first meiotic division it is switched on by the maturing oocyte itself. It would seem, therefore, that in the absence of an intrinsic factor triggering first anaphase, the agents known to activate the mature egg might substitute for it. However, in normal course of maturation (with the exception of some limited ability described in the CBA strain; Polanski, 1990), MI mouse oocytes are not capable of becoming activated (Iwamatsu and Chang, 1972; Clarke and Masui, 1986; McConnell *et al.*, 1995). This capacity appears in MII oocytes as their postovulatory age increase (Kaufman, 1973; Kubiak, 1989). Indeed, the MI-arrested F_1 oocytes in this study only acquired their ability to undergo activation later, and developed it gradually, in parallel to MII oocytes. This suggests that in spite of arrested nuclear maturation in MI oocytes, their cytoplasm matured reaching to a state at which they may respond to appropriate external activating stimuli. Moreover, the difference between CBA and KE strains in the susceptibility of MI oocytes to respond to spermatozoa reflects the difference between the MII oocytes from these strains. In addition, the total frequency of activation of oocytes from F_1 mice does not differ significantly between MI and MII oocytes. This suggests a considerable similarity between the functional state of the cytoplasm from MI-arrested and MII oocytes.

Recently, whilst the results presented in this study were being prepared for publication, similar data were published by Eppig *et al.* (1994). These authors showed that MI-arrested oocytes upon insemination extruded PB1, formed pronuclei, cleaved and developed to the blastocyst stage. MI-arrested oocytes treated with calcium ionophore also underwent parthenogenetic activation and the pattern of protein synthesis in the MI-arrested oocytes was similar to that in MII oocytes. These authors concluded that cytoplasmic maturation, or at least some of its critical aspects, can occur in mouse oocytes whose nuclear maturation is arrested at MI (Eppig *et al.*, 1994). The uncoupling of cytoplasmic and nuclear maturation in mouse oocytes was also demonstrated more recently by McConnell *et al.* (1995). This study, using mice of a different genetic origin, confirms the possibility of parthenogenetic activation of MI-arrested oocytes and gives additional evidence for the similarity between the cytoplasm of MI-arrested and MII oocytes. Thus, the ability to undergo activation in first meiotic metaphase is not exclusive to LT/Sv oocytes bearing a unique mutation (West *et al.*, 1993) but, in certain conditions (after incomplete maturation *in vitro*), it may be a common feature of mouse oocytes regardless of their genetic composition.

As documented in Table 4, in MII oocytes cultured for an equivalent time, the activation ability increased with the time elapsed since PB1 extrusion. This shows that, independently of the cytoplasm maturity, the oocyte needs some resting time after PB1 extrusion before it can be activated. Kubiak *et al.* (1992) reported that formation of the second metaphase spindle starts 60-75 min after PB1 extrusion and the high level of MPF is re-established even later, about 2.5 h after PB1 extrusion. Thus, such a resting time seems to be necessary to restore a correct balance between factors of the cell cycle control machinery, following their involvement in the first meiotic division. This would also explain why among MII oocytes, in contrast to MI oocytes, many activated abortively.

In mammals vast numbers of small primary oocytes are present in the ovary from before birth, however only a small portion of them are used by the female throughout her reproductive life. Thus, primary oocytes are a potentially large source of material for experimental or zootechnical purposes. There is an increasing number of reports of the successful maturation *in vitro* of primary or preantral oocytes after their growth in culture (Eppig and Schroeder, 1989; Hirao *et al.*, 1990; Carroll *et al.*, 1991). Under these conditions, however, the number of oocytes attaining an MII status may be reduced. For example, of 40% of oocytes undergoing GVB, half blocked subsequently in MI (Carroll *et al.*, 1991). However, this study shows that such oocytes may be activated either fully, entering first mitotic interphase, or abortively, stopping at the second meiotic metaphase. It would be tempting to speculate the development of a system for abortive activation of such oocytes, which might improve efficiency of maturation in cases where it is reduced due to MI arrest.

Materials and Methods

In vitro oocyte maturation

Adult females from the inbred mouse strains KE and CBA or from their F_1 crosses (in both directions) were killed by cervical dislocation regardless of the stage of the estrus cycle. The ovaries were dissected

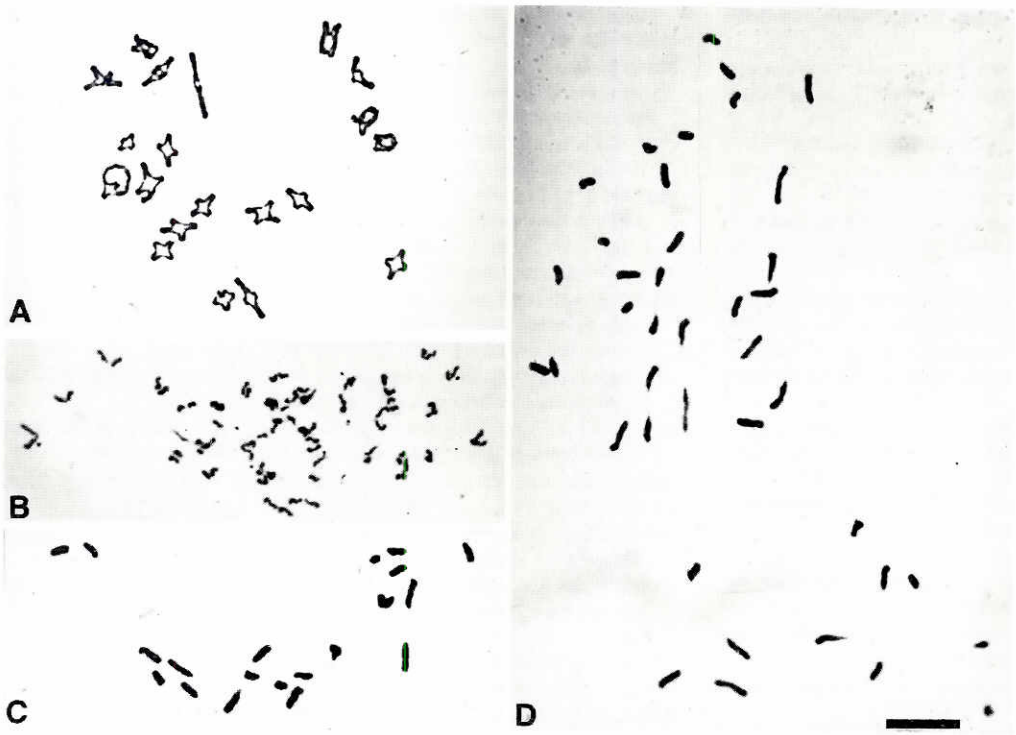

Fig. 2. Detailed chromosome examination of oocytes after maturation in culture (A,B) and oocytes which, after maturation *in vitro*, were fully activated by electric shock treatment and cultured for one day (C,D). (A) *20 divalent chromosomes from control MI-arrested oocyte.* **(B)** *≈40 monovalent chromosomes from a control oocyte which progressed to MII without extrusion of PB1.* **(C)** *20 mitotic chromosomes from an oocyte activated in MII (after PB1 extrusion).* **(D)** *40 mitotic chromosomes from an oocyte activated in MI. Staining with 2% Giemsa solution. Bar, 10 μM.*

and placed in M2 culture medium (Fulton and Whittingham, 1978). Oocytes were released into the medium by puncturing antral ovarian follicles. The cumulus cells were removed mechanically and oocytes at the germinal vesicle (GV) stage were collected. Zonae pellucidae were removed from oocytes to be inseminated by brief exposure to a 0.05% solution of α-chymotrypsin (Sigma Chemical Co.). This was necessary because oocytes matured in medium without serum become impenetrable to spermatozoa due to hardening of the zona pellucida (Downs *et al.*, 1986). Finally, after rinsing 3 times in M2, the oocytes were cultured in this medium at 37°C for 20 h (for insemination) or 14, 16 and 18 h (for parthenogenetic activation). At the end of the assigned culture period, oocytes were analyzed for the progression of meiotic maturation. Oocytes still at the GV stage were discarded. Oocytes in MI and MII, as indicated by the absence or presence of PB1, were inseminated or parthenogenetically activated.

Fertilization in vitro

Spermatozoa were collected from the cauda epididymis and vasa deferentia of adult CBA males into a 0.4 ml drop of fertilization medium (Toyoda *et al.*, 1971) and incubated for 70-90 min at 37°C in an atmosphere of 5% CO_2 in air to allow for capacitation. Oocytes were rinsed and placed in drops of fertilization medium to which aliquots of preincubated sperm suspension were added. The final concentration of spermatozoa in different experiments ranged between $1x10^4$ and $2.5x10^5$. After incubation with spermatozoa for 1 h, the oocytes were washed and incubated for a further 3 h in fertilization or M16 medium (Whittingham, 1971) before fixation. Control (non-inseminated) oocytes were processed in the same way as experimental ones except that no spermatozoa were added to fertilization drops.

Parthenogenetic activation

Oocytes were activated by electric shock in an electrofusion chamber. The chamber was filled with common electrofusion solution of 0.25 M glucose in water, supplemented with a small amount of M2 (100 μl M2/3 ml glucose solution). Oocytes were placed between two parallel platinum wire electrodes (1 mm apart) and treated with an electric current (DC, 100V, duration 25 μs, two pulses 100 ms apart). They were then washed 3 times and cultured in M2 for 5-6 h before fixation.

Oocyte fixation

Oocytes were processed for air dried preparations and stained with toluidine blue according to Krzanowska and Lorenc (1983), which permits penetrated ova to be easily demonstrated. Only sperm heads which entered the vitellus became stained, whether the chromatin starts to decondense or remains in a condensed state (Krzanowska and Lorenc, 1983). Chromosome preparations were made according to the method of Tarkowski (1966).

Statistical analysis

The data were analysed using the χ^2 test of independence adjusted for low numbers (Snedecor, 1955), or one-way analysis of variance (ANOVA).

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