

## Genetically identical parthenogenetic mouse embryos produced by inhibition of the first meiotic cleavage with cytochalasin D

JACEK KUBIAK<sup>1,‡</sup>, ANDRAS PALDI<sup>2,\*</sup>, MICHÈLE WEBER<sup>1</sup> and BERNARD MARO<sup>1,†</sup>

<sup>1</sup>Laboratoire de Physiologie du Développement

<sup>2</sup>Laboratoire de Génétique Physiologique,

Institut Jacques Monod, UMR3 CNRS-Paris 7 and U257 INSERM, Tour 43-2, place Jussieu, 75005 Paris, France

\*Permanent address: Agricultural Biotechnology Center, 2101 Gödöllő pf. 170, Hungary

† Author for correspondence

‡ On leave from: Department of Embryology, Institute of Zoology, Warsaw University, Poland

### Summary

The microfilament inhibitor cytochalasin D inhibits extrusion of the first polar body when present during the first meiotic division of mouse oocytes; however, it does not interfere with anaphase movement of chromosomes, and thus induces the formation of tetraploid oocytes. After the separation of chromosomes in anaphase, two spindles start to assemble. However, they merge rapidly and a single meiotic spindle forms. During the transition between metaphase I and metaphase II, in the presence of cytochalasin D, a drop in histone kinase activity takes place demonstrating a transitional decrease in the activity of the maturation promoting factor. These oocytes can be activated parthenogenetically a few hours after washing out the inhibitor. After completion of the second meiotic division and extrusion of a polar body, they contain a diploid number of chromosomes. They

are genetically identical to each other and to their mother. Such eggs develop to the blastocyst stage and can implant in the uteri of foster mothers. Most of these fetuses die before the 9th day of gestation, as do diploid control fetuses treated with cytochalasin D during the second meiotic division. The heterozygous state of the experimental embryos obtained after activation of eggs recovered from heterozygous females and treated with cytochalasin D during the first meiotic division was confirmed using a glucose-phosphate isomerase assay. This technique allows the production of genetic clones of parthenogenetic embryos by simple means.

Key words: cell cycle, cytochalasin, parthenogenesis, mouse oocyte, spindle, microtubule.

### Introduction

Cytochalasins are potent inhibitors of actin microfilaments and have been used to increase the ploidy of various cells. The presence of cytochalasin during cell division does not disturb the anaphase movement of chromosomes (karyokinesis), but inhibits the separation of daughter cells (cytokinesis), both sets of chromosomes remaining in a single cell, doubling its ploidy. Cytochalasin B has been used for the diploidisation of mouse oocytes activated parthenogenetically *in vitro* by blocking second polar body extrusion (Balakier and Tarkowski, 1976). It can also be used to produce tetraploid embryos (Snow, 1973), or even higher ploidy depending on number of cytokineses blocked (Petzoldt *et al.* 1983). During all these cell divisions, as for normal somatic cell mitosis, the cells undergo transition to interphase and form two interphase nuclei, which merge during the subsequent metaphase. Hashimoto and Kishimoto (1988) have applied cytochalasin D

(CCD) during the first meiotic division of the mouse oocyte (i.e. at the transition between the first and second meiotic metaphase, MI and MII respectively, which proceeds normally without formation of any interphase nucleus (Donahue, 1968)) and suggested that this drug blocks not only the first polar body extrusion, but also stabilizes maturation promoting factor (MPF) activity at high, metaphase levels. They also demonstrated that during the normal MI–MII transition the activity of MPF drops to the low level characteristic for interphase. Instead of 20 bivalents characteristic for MI, they found a single metaphase spindle with 40 monovalent chromosomes in cytochalasin D-treated oocytes after the time of the transition, and concluded that cytochalasin D allows splitting of the bivalents, but prevents anaphase I movement of monovalents during the first meiotic division. Since this cell division would be the only example of cytochalasin D-arrested karyokinesis, we have reexamined this phenomenon using immunofluorescence microscopy of

tubulin and staining of chromatin. In this paper, we show that after cytochalasin D treatment oocytes are able to resume the normal course of meiosis and have the same developmental potential as classical diploid parthenogenotes.

## Materials and methods

### Recovery of oocytes

Mouse oocytes were recovered from the ovaries of  $F_1$ (C57Bl/DBA2),  $F_1$ (C57Bl/CBA) or Swiss females 7–8 h after hCG injection (i.e. 2–3 h before the first meiotic division). The experimental protocol is presented in Fig. 1. The experimental group of oocytes (CCD+–) was cultured in M2 medium containing  $1 \mu\text{g ml}^{-1}$  cytochalasin D (CCD), while the control group (CCD–+) was cultured in M2 medium alone. After 3–4 h, i.e. when the oocytes in the control group (CCD–+) had completed first polar body extrusion, the drug was washed out from the medium in which the experimental eggs were being incubated and the oocytes were further cultured in M2. Both groups were activated by treatment with 8% ethanol in M2 for 6 min between 16 and 20 h post-hCG (Cuthbertson, 1983; Kaufman, 1983). The experimental group (CCD+–) was allowed to undergo normal extrusion of a polar body in M2, while the control group (CCD–+) was cultured for 3–4 h in the presence of  $1 \mu\text{g ml}^{-1}$  cytochalasin D in M2 to prevent extrusion of the second polar body. Activated oocytes were transplanted into the oviducts of randomly chosen females for 3 days or cultured overnight *in vitro*

*in vitro* and transplanted after the first cleavage division into the oviducts of pseudopregnant females during the first day *post coitum*. The foster mothers were killed on the 9th or 10th day of gestation, and their uteri were examined for presence and morphology of fetuses.

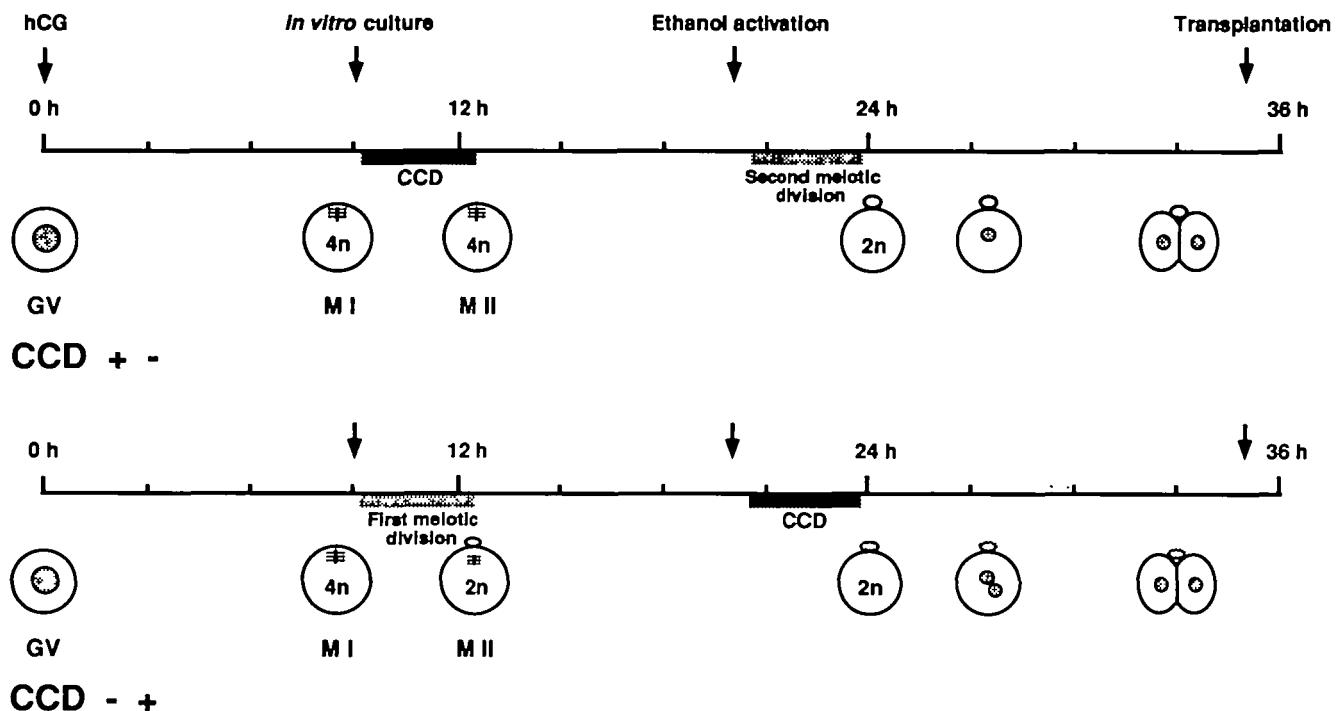
### Immunofluorescence

The *zonae pellucidae* were removed by brief treatment with acid Tyrode's solution, pH 2.5 (Nicolson *et al.* 1975). Oocytes were placed in specially designed stainless steel chambers as described by Maro *et al.* (1984) except that the chambers were coated with  $0.1 \text{ mg ml}^{-1}$  concanavalin A (Sigma). The samples were centrifuged at 450 g for 8–10 min at  $37^\circ\text{C}$ , and after centrifugation fixed with 0.1% glutaraldehyde (Sigma) in PBS supplemented with 1% Triton X-100 (Boehringer Mannheim GmbH) as described by de Pennart *et al.* (1988). After a 5 min wash in PBS, they were extracted with 2% Triton X-100 for 30 min, incubated in  $10 \text{ mg ml}^{-1}$   $\text{NaBH}_4$  in PBS (three incubations of 10 min each), washed twice in PBS and processed for immunofluorescence as described by Maro *et al.* (1984).

We used rat YL1/2 antibody specific for tyrosinated alpha-tubulin (Kilmartin *et al.* 1982). Fluorescein-labelled anti-rat antibody (Miles) was used as a second layer. For visualizing chromatin, DAPI ( $5 \mu\text{g ml}^{-1}$  in PBS) was included in the last wash of the specimens.

### Histone H1 kinase assay

Histone H1 kinase activity was determined as described by Felix *et al.* (1989) in HK buffer ( $80 \text{ mM } \beta\text{-glycerophosphate}$ ,



**Fig. 1.** Experimental protocol used in this study. Mouse oocytes were recovered from the ovaries 7–8 h after hCG injection. The experimental group of oocytes (CCD+–) was cultured in medium containing cytochalasin D, while the control group (CCD–+) was cultured in medium alone. After 3 h, i.e. when the oocytes in the control group (CCD–+) had completed first polar body extrusion, the drug was washed out and the oocytes were further cultured in medium. Both groups were activated by treatment with ethanol at about 18 h post-hCG. The experimental group (CCD+–) was allowed to undergo normal extrusion of a polar body, while the control group (CCD–+) was cultured for 3 h in the presence of  $1 \mu\text{g ml}^{-1}$  cytochalasin D to prevent extrusion of the second polar body. When necessary, activated oocytes were cultured overnight *in vitro* and transplanted after the first cleavage division into the oviducts of pseudopregnant females.

20 mM EGTA pH 7.3, 15 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM PMSF, 10 µg ml<sup>-1</sup> leupeptin, 10 µg ml<sup>-1</sup> pepstatin, 10 µg ml<sup>-1</sup> aprotinin) using exogenous histone H1 (HIII-S from calf thymus, Sigma) as substrate. Samples each containing 50 oocytes in 5 µl of water were lysed by freezing and thawing three times, diluted twice in two times concentrated HK buffer (2×HK) and incubated for 15 min at 20°C in the presence of 3.3 mg ml<sup>-1</sup> histone H1, 1 mM ATP and 0.25 mCi ml<sup>-1</sup> [<sup>32</sup>P]ATP. The reaction was stopped by the addition of a similar volume of two times concentrated sample buffer (Laemmli, 1970) and incubation for 2 min at 90°C. The samples were then electrophoresed on a 15% SDS-polyacrylamide gel (Laemmli, 1970). In order to test the specificity of the reaction, the p34<sup>cdc2</sup> kinase (histone H1 kinase) was removed by centrifugation from the control sample using p13<sup>suc1</sup>-coated sepharose beads (gift of M. Dorée).

#### GPI assay

The assay was performed according to the method described by McLaren and Buehr (1981).

## Results

### *Cytochalasin D inhibits first polar body extrusion, but not anaphase I movements*

In the presence of cytochalasin D, the first meiotic division takes place, but cleavage is impaired and the first polar body is not extruded (Fig. 2A,B). Two separate spindles start to form around each group of chromosomes (Fig. 2C,D). The midbody microtubules, which separate the two groups of chromosomes, disassemble soon after anaphase (Fig. 2C), and both sets of chromosomes surrounded by microtubules merge to form a single metaphase plate (Fig. 2D,E). During the merging of the two spindles, the chromosomes are dispersed within the spindle volume indicating that spindle formation is not yet completed (Fig. 2D). Later on, the chromosomes form a well-organized metaphase plate within a compact, well-formed spindle between 1–2 h after anaphase I (Fig. 2E,F). This spindle is equivalent to the second meiotic spindle (MII); however, it contains a tetraploid number of chromosomes instead of a diploid one.

### *Histone H1 kinase activity drops during anaphase I in presence of cytochalasin D*

The histone H1 kinase activity reflects the level of the maturation promoting factor (MPF) activity and is due to the p34<sup>cdc2</sup> protein, homolog of the product of the gene *cdc2* in *Schizosaccharomyces pombe* (Draetta and Beach, 1988; Dunphy *et al.* 1988; Gautier *et al.* 1988; Labbe *et al.* 1988, 1989). We measured the activity of histone H1 kinase in the lysate of F<sub>1</sub>(CBA/C57Bl) oocytes before (MI), during (anaphase I), and after (MII) the transition in the presence of cytochalasin D. Since, in the presence of the drug, the stage of karyokinesis is not visible in living oocytes due to the absence of polar body extrusion, we used 500 ng ml<sup>-1</sup> Hoechst 33342 to visualize chromosomes under a fluorescent microscope. Using this technique, we selected oocytes undergoing anaphase I and either lysed

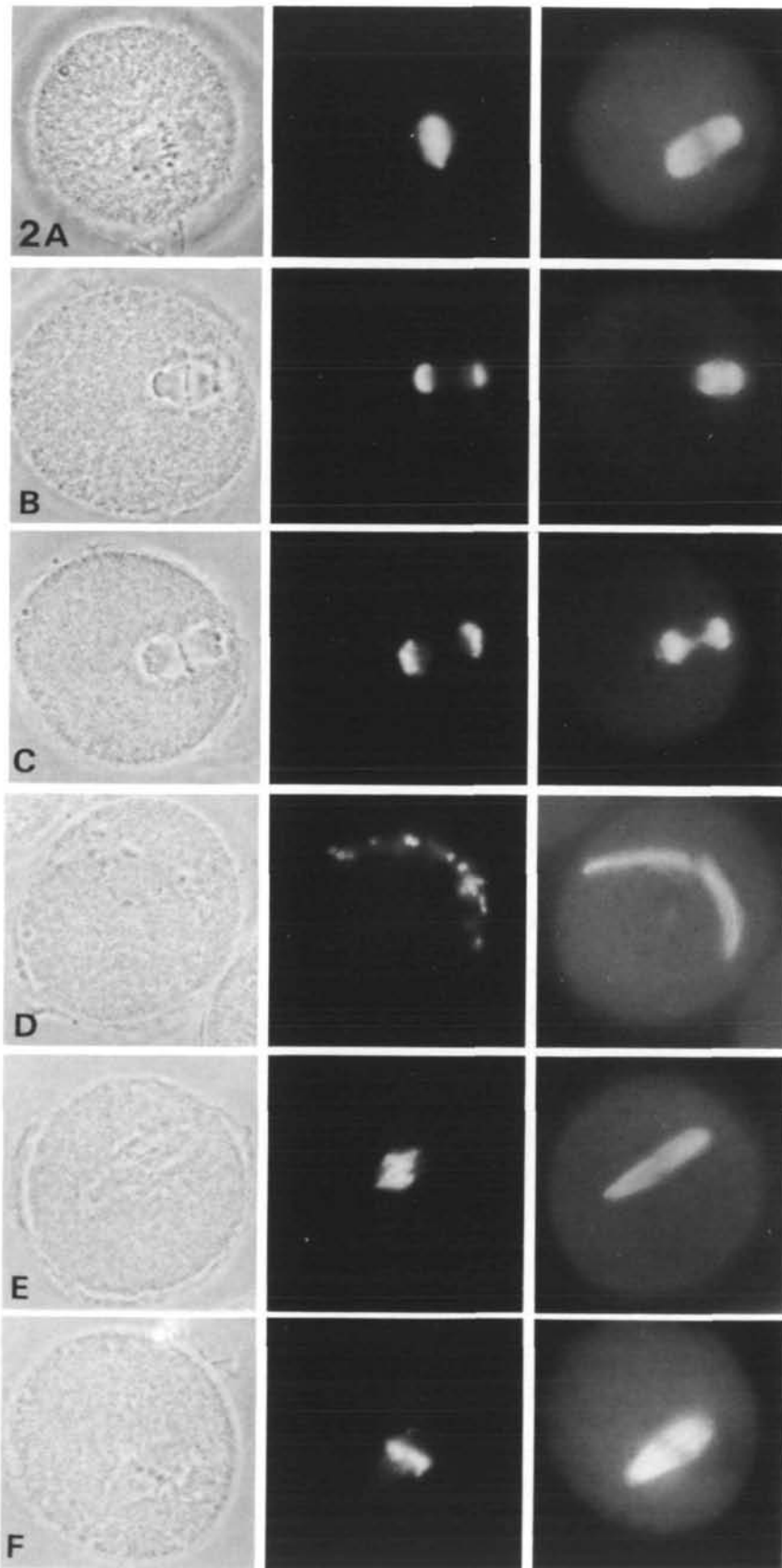
them immediately or allowed them to complete karyokinesis, culturing for a further hour. The MI oocytes were arrested in the M-phase by 5 µM nocodazole added to the cytochalasin D-containing medium. The *in vitro* measurement of histone kinase activity in the lysates of these three groups of oocytes revealed a significant drop of this activity during the transitional period despite the presence of cytochalasin D (Fig. 3). The specificity of the reaction was tested in a control experiment where a lysate of MII oocytes was incubated with sepharose beads coated with p13<sup>suc1</sup>, a protein that binds specifically to p34<sup>cdc2</sup> (Draetta *et al.* 1987). This allowed us to remove p34<sup>cdc2</sup> from the lysate. After centrifugation the supernatant was used in the assay and served as a reference point to estimate the histone H1 activity in the experimental groups (Fig. 3). During the normal transition between MI and MII, using polar body extrusion as a reference point, we observed a more dramatic drop in histone kinase activity, characteristic for interphase (Kubiak, Weber and Maro, data not shown). The difference observed between the levels of H1 kinase activity during the MI–MII transition in the absence and the presence of cytochalasin D is caused by a poorer synchrony of the cytochalasin D-treated oocytes, since the time interval necessary for handling oocytes under a fluorescent microscope is much longer and in consequence some of the oocytes in the sample of anaphase oocytes could already have entered early MII.

### *Activation of tetraploid oocytes results in extrusion of the polar body and reconstitution of a diploid number of chromosomes*

Oocytes that had the first meiotic cleavage suppressed by cytochalasin D were able to respond to an activating stimulus a few hours later (Fig. 4). In most cases, they extruded a polar body and formed a single pronucleus. Others underwent immediate cleavage, or abortive activation (extrusion of a polar body and reentry in a subsequent metaphase; (Kubiak, 1989)). The relative percentages of oocytes reacting in these ways depended on the age of the oocytes at the time of activation. A high proportion of oocytes activated soon after the MI–MII transition (17 h post-hCG) reacted by abortive activation, while later activation (21 h post-hCG) resulted in a normal response, i.e. pronuclear formation (either one or two pronuclei), as well as the appearance of some immediately cleaving oocytes (Table 1). These results show that the oocytes reach a stage physiologically equivalent to MII, despite the presence of cytochalasin D during the first meiotic division.

### *F<sub>1</sub>(C57Bl/DBA2) parthenogenetic embryos obtained by inhibition of the first meiotic division are heterozygous for the glucose-phosphate-isomerase (GPI) locus*

Both the experimental (CCD+–) and control (CCD–+) groups of embryos obtained from F<sub>1</sub>(C57Bl/DBA2) females heterozygous at the GPI locus were either cultured *in vitro* or transplanted into

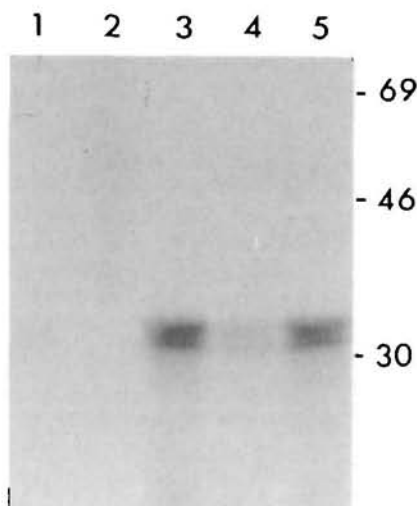


**Fig. 2.** Cytological analysis of the M1-MII transition in the presence of cytochalasin D: phase contrast (left panel), hoechst labelling (central panel) and anti-tubulin labelling (right panel). (A) Metaphase I; (B) anaphase I; (C) telophase I; (D) early prometaphase II; (E) late prometaphase II; (F) metaphase II. Note that, in the presence of cytochalasin D, the first meiotic division takes place, but cleavage is impaired and the first polar body is not extruded. Two separate spindles start to form around each group of chromosomes, then both spindles merge to form a single metaphase II spindle. Note, that chromosomes remain condensed during the whole transition.

**Table 1.** Activation of (CCD+-) and (CCD-+) oocytes at different ages after hCG injection

	<i>n</i>	Metaphase II (%)	Metaphase after abortive activation (%)	Two pronuclei (%)	Single pronucleus with second polar body (%)	Immediate cleavage (%)	Single pronucleus without second polar body (%)
1. Activation 17 h post-hCG							
(CCD+-)	400	45	39.5	—	15.5	—	—
(CCD-+)	174	58.5*	—	41.5	—	—	—
2. Activation 21 h post-hCG							
(CCD+-)	290	7.5	4.5	—	76.5	10	1.5
(CCD-+)	86	18*	—	82	—	—	—

\*Metaphase II oocytes and metaphase oocytes after abortive activation together, since they are not easily distinguishable.

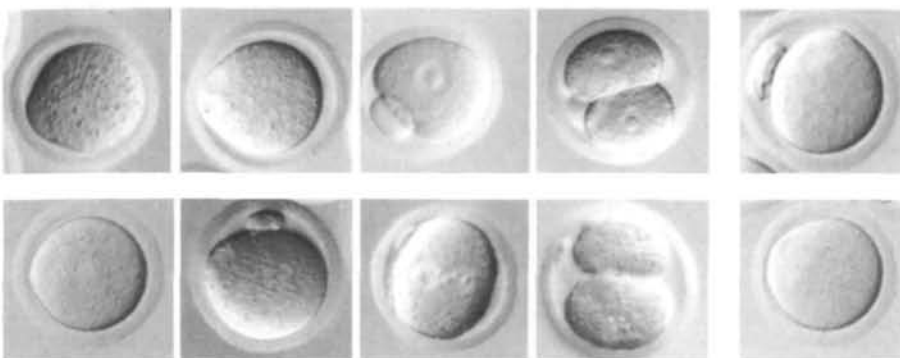


**Fig. 3.** Histone H1 kinase activity during the MI-MII transition in the presence of cytochalasin D. Autoradiograms of  $^{32}\text{P}$ -phosphate incorporated into histone H1. Lane 1: control, the lysate of MII oocytes used to phosphorylate histone H1 was first incubated with sepharose beads coated with  $\text{p}13^{\text{suc}1}$  and then centrifuged to remove  $\text{p}34^{\text{cdc}2}$  from the lysate; lane 2: control, lysate from metaphase I oocytes, no exogenous histones; lane 3: lysate from metaphase I oocytes; lane 4: lysate from anaphase I oocytes cultured in the presence of cytochalasin D; lane 5: lysate from metaphase II oocytes cultured in the presence of cytochalasin D. The position of the relative molecular mass markers is indicated on the right of the autoradiogram.

**Table 2.** Glucose-phosphate isomerase analysis of individual parthenogenetic blastocysts

	Genotype		
	A/A	A/B	B/B
(CCD+-)	0	32 (100%)	0
(CCD-+)	9 (26%)	6 (17%)	20 (57%)

oviducts of random-bred females for three days to test their viability. They underwent similar development up to the blastocyst stage in both conditions. Since we were able to obtain a greater number of well-developed blastocysts using the *in vivo* technique, we used these embryos to test their GPI constitution. We made an electrophoretic analysis of single expanded blastocysts on their fifth day of development. All successfully tested blastocysts ( $n=32$ ) from the experimental group (CCD+-) show three bands corresponding to the three isoforms of the enzyme (A/A, A/B and B/B) characteristic for heterozygotes. 83% of blastocysts in the control group (CCD-+) ( $n=35$ ) were homozygous for this locus. The heterozygotes A/B (17%) represent blastocysts developed from oocytes in which the GPI locus underwent recombination (Table 2). Haploid blastocysts ( $n=20$ ) of the same strain and age obtained by activation of the control oocytes without cytochalasin D show only one form (A or B) of the enzyme indicating disappearance of the maternal isozymes at the specified period of development.



**Fig. 4.** Morphology of oocytes which had the first (CCD+-; top panel) or the second (CCD-+; bottom panel) meiotic cleavage suppressed by cytochalasin D from metaphase I to the 2-cell stage. From left to right: metaphase I, metaphase II, pronuclear stage, 2-cell stage and abortive activation with extrusion of a polar body and reentry in metaphase.

**Table 3.** Postimplantation development of parthenogenetic embryos

	Day of pregnancy	Number of			viable embryos (% implantation)
		recipients	transplanted eggs	implantations	
(CCD+-)	9	5	56	43 (77%)	8 egg cylinders and 3 pre-somite stage fetuses (26%)
(CCD+-)	10	8	122	60 (49%)	1 well developed fetus (12 somites) (2%)
(CCD-+)	9	8	96	68 (71%)	18 egg cylinders (26%)
(CCD-+)	10	8	94	60 (64%)	(0%)

*Heterozygous, diploid parthenogenotes of F<sub>1</sub>(C57Bl/DBA2) strain develop similarly to homozygous, diploid ones*

Activated (CCD+-) oocytes which extruded the polar body and formed a single pronucleus (potentially diploid heterozygotes), as well as control oocytes (CCD-+), which underwent the second meiotic division in the presence of cytochalasin D, were transplanted to the oviducts of pseudopregnant females. The implantation rate was around 70% in both groups, a percentage similar to the one observed with normal control fertilized embryos. The foster mothers were killed on the 9th or 10th day of gestation. Most of the parthenogenetic fetuses in both groups had degenerated by day 9 (Table 3). The surviving embryos from both groups recovered at this age were poorly developed and resembled egg-cylinder-stage embryos of day 7 of normal development except for one well developed fetus with 12 pairs of somites and a beating heart in the experimental group (CCD+-) on day 10.

## Discussion

Previous data published by Hashimoto and Kischimoto (1988) suggested that, in contrast to other cell divisions, the first meiotic division is arrested by cytochalasin D at both the karyokinesis and cytokinesis levels. As the first meiotic division proceeds, a particular modification of the cell cycle takes place, namely one metaphase (MI) is directly followed by another metaphase (MII). The period between these two metaphases represents, in fact, a very short interphase, short enough to prevent interphase nucleus formation. Since we know of no other data showing an interference with anaphase movements by cytochalasin D, we were intrigued by the reported exceptional action of this drug on this particular transition. Our results demonstrate that cytochalasin D acts similarly during the first meiotic division of mouse oocytes, during the second meiotic division and during mitosis. However, the result of cytochalasin D presence during the first meiotic division i.e. formation of a single metaphase plate, was to some extent unexpected, and was probably the reason for the previous misinterpretation of the action of this drug (Hashimoto and Kishimoto, 1988). The merging of both groups of chromosomes is possible due to the rapid

disassembling of midbody microtubules, which takes place before definitive formation of the spindles. In the case of the second meiotic division, as well as during mitotic divisions, the transition to interphase takes place, and midbody microtubules persist for a prolonged time (Kidder *et al.* 1988; Kubiak, Weber and Maro, data not shown). A similar sequence of events occurs in cells treated with cytochalasin D. When the first meiotic division of mouse oocytes takes place in the presence of cytochalasin D and 20  $\mu$ M puromycin (an inhibitor of protein synthesis causing transition of the oocytes to interphase), two nuclei separated by midbody microtubules formed in all oocytes treated with both drugs. This demonstrates that midbody microtubule behaviour does not depend on the presence or absence of cytochalasin D, but on cell-cycle-dependent modifications of cytoplasmic conditions.

The activity of histone H1 kinase drops during the MI-MII transition in the presence of cytochalasin D as it does in the normal MI-MII transition (Kubiak, Weber and Maro, data not shown). This represents a change in the activity of the p34<sup>cdc2</sup> protein kinase, which is the active part of MPF (Draetta and Beach, 1988; Dunphy *et al.* 1988; Gautier *et al.* 1988; Labbe *et al.* 1988, 1989). Thus, the drop in this activity reflects the inactivation of MPF during the transitional period in the oocytes despite the continuous presence of cytochalasin D. These results remain in conflict with those of Hashimoto and Kischimoto (1988) that suggested that cytochalasin D stabilizes the MPF activity in a similar manner to colcemid, but this could be explained by the difficulty of getting well-synchronized samples in the presence of cytochalasin D.

The normal course of meiosis with respect to the physiological properties of oocytes is also demonstrated by their ability to undergo activation even after the inhibition of first polar body extrusion by cytochalasin D. Oocytes in MI can not be activated (Clarke and Masui, 1983; Iwamatsu and Chang, 1972) except in the case of the LT/Sv strain (Kaufman and Howlett, 1986), and this ability develops at the beginning of the MII arrest (Kubiak, 1989). Since we observed a similar sequence of events in cytochalasin D-treated oocytes after washing out the drug, it demonstrates that they are indeed at a stage physiologically equivalent to MII.

The experimental protocol presented in this paper allowed us to obtain tetraploid oocytes, which could be

activated to produce parthenogenetic embryos identical to each other and to their mothers – a true genetic clone. These embryos retain such properties since their genomes contain a single copy of each chromatid of every maternal monovalent chromosome. This means that, even in the case of recombined genes after crossing-over, the resulting embryonic genome represents a clone of the mother, only with the genes differently arranged on the sister chromatids. This effect could not be obtained using other methods of producing parthenogenetic embryos.

The cytochalasin D arrest of second polar body extrusion results in the formation of diploid embryos that have various gene compositions, since only a half of the original maternal chromosomes enter their genome. In addition, the majority of embryonic genes are in a homozygous state, with the exception of those that underwent crossing over. In contrast, the inhibition of first polar body extrusion by cytochalasin D provides an experimental method of creating diploid heterozygous, parthenogenetic embryos (with a genome similar to the genome of the mother).

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