Int. J. Dev. Biol. 35: 421-427 (1991)



*Original Article*

# Cell cycle-dependent behavior of microtubules in hybrids of mouse oocytes and blastomeres[1]

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**ABSTRACT The behavior of microtubules was studied in hybrids formed between mouse oocytes arrested in metaphase II or activated parthenogenetically and mouse embryo interphase blastomeres. In all cases the interphase blastomere's network of microtubules disassembles rapidly after fusion with oocytes. Introduction of interphase cytoplasm and nuclei to metaphase oocytes during fusion induces the polymerization of new microtubules in the cytoplasm and in the meiotic spindle. The degree and the duration of this facilitated polymerization of microtubules was positively correlated with the volume of blastomeres used for fusion. The blastomere nuclei induce the formation of microtubular frames, which become more evident when the chromatin undergoes premature condensation. Finally, spindle-like structures are formed around the prematurely condensed chromosomes. In hybrids activated around the time of fusion, the blastomere nuclei undergo pronuclear-like transformation. These hybrids develop an interphase network of microtubules typical for activated oocytes. These results are discussed with regards to the cell cycle control of microtubule behavior.**

KEY WORDS: *blastomeres, cell cycle control, electrofusion, microtubules, mouse, oocyte activation*

## Introduction

The cell cycle in all eukaryotic cells is controlled by a common mechanism involving the activation of the M-phase promoting factor (MPF) during the interphase/M-phase transition and its inactivation during M-phase/interphase transition (Masui and Markert, 1971; Gerhart *et al.*, 1984; Hashimoto and Kishimoto, 1988). MPF was identified as a complex of two proteins: cyclin as the regulatory subunit and a homolog of yeast *Schizosaccharomyces pombe* cdc2 gene product as the catalytic subunit with a protein kinase activity (reviewed by Dunphy *et al.*, 1988; Murray, 1988; Lohka, 1989; Nurse, 1990). At least two cyclins (A and B) seem to be involved in the regulation of the kinase activity of the complex during G2/M/G1 transition in mammalian cells (Pines and Hunter, 1989; Pines and Hunter, 1990; Pines and Hunter, 1991). Cyclin B forms a complex with $p34^{cdc2}$, while cyclin A associates not only with the latter, but also with another cdc2-related protein, p33 (reviewed by Hunter and Pines, 1991). Activation and inactivation of cdc2 kinase is mediated not only by association of $p34^{cdc2}$ and/or cdc2-related proteins with cyclins, but also by dephosphorylation and phosphorylation of specific sites on p34cdc2 molecule (Gould *et al.*, 1991; Krek and Nigg, 1991; Norbury *et al.*, 1991), while there are no similar data available on the state of phosphorylation of p33. Nuclear envelope disassembly and chromosome condensation is triggered by the cdc2 kinase as the cell enters M-phase (Gautier *et al.*, 1988; Lohka *et al.*, 1988). The activity of this protein kinase can be measured *in vitro* by phosphorylation of exogenous histone H1 (Picard *et al.*, 1987; Gautier *et al.*, 1988; Labbé *et al.*, 1989). The presence of high activity of MPF (Hashimoto and Kishimoto, 1988), and of histone H1 kinase (Rime and Ozon, 1990; Kubiak *et al.*, 1991a) was demonstrated in mouse oocytes during the meiotic M-phases. Fusion of metaphase and interphase cells induces premature chromatin condensation (PCC) of the interphase nucleus as the result of the MPF activity derived from the M-phase fusion partner (Johnson and Rao, 1970). A similar reaction is observed in hybrids obtained between mouse oocytes (M I or M II) and interphase cells (Balakier and Czolowska, 1977; Balakier, 1978; Tarkowski and Balakier, 1980; Czolowska *et al.*, 1984; Czolowska *et al.*, 1986; Szöllösi *et al.*, 1986b). Fertilization or parthenogenetic activation of oocytes induces the transition to interphase and the rapid disappearance of MPF activity. This is shown by the inactivation of histone H1 kinase (Rime and Ozon, 1990; Weber *et al.*, 1991), degradation of cyclin B (Weber *et al.*, 1991) and disappearance of PCC-inducing activity (Tarkowski and Balakier, 1980; Czolowska *et al.*, 1984; Szöllösi *et al.*, 1986a; Szöllösi *et al.*, 1988).

*Abbreviations used in this paper*: Cc, critical concentration; MPF, maturation promoting factor; PCC, premature chromatin condensation; PCM, pericentriolar material.

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[1]*This paper is dedicated to Daniel Szöllösi.*

0214-6282/91/$03.00



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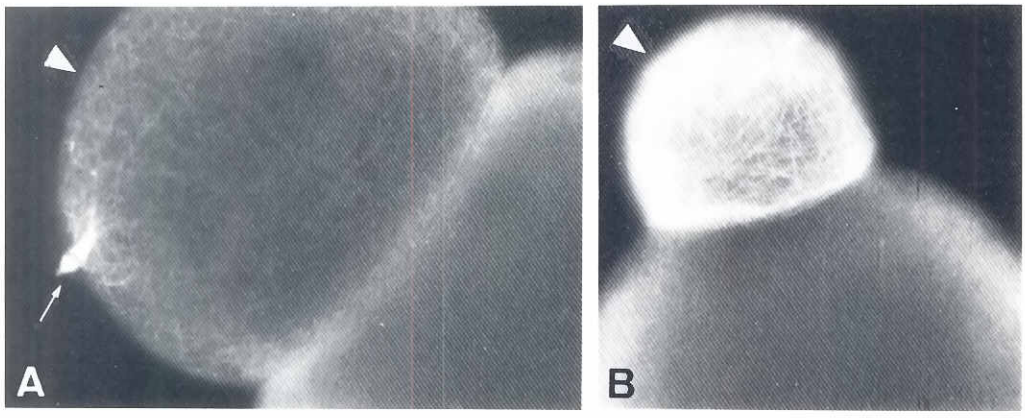


**Fig. 1. Control pairs of blastomeres (arrowheads) and oocytes (stars) which failed to fuse. (A and B) Tubulin staining**. *Single 2-cell embryo blastomere* **(A)** *and 8-cell embryo blastomere* **(B)** *agglutinated with oocytes. Note that the microtubule network in the 2-cell embryo blastomere is much more delicate then in the 8-cell stage. Arrow indicates midbody microtubules remaining at the surface of the 2-cell embryo blastomere after separation from its sister blastomere. The microtubule network is denser in the vicinity of the midbody than anywhere else in the blastomere.*

The nuclear changes taking place during the cell cycle transitions between M-phase and interphase are associated with rearrangements of the cytoskeleton. Microtubules, which represent the most dynamic cytoskeletal elements, form a metaphase spindle during M-phase and an interphase network during interphase. The different characteristics of microtubules in M-phase and in interphase are mediated by the cdc2 kinase (Verde *et al.*, 1990), probably via phosphorylation of certain microtubule-associated proteins (MAPs), as XMAP in *Xenopus laevis* oocytes and eggs (Gard and Kirschner, 1987a,b). In the presence of active cdc2 kinase (M-phase) the growth of microtubules is restricted, while inactivation of this kinase (interphase) favors microtubule elongation (Verde *et al.*, 1990). In other words, during the M-phase the critical concentration (Cc) for tubulin polymerization is high, while it is lowered in the interphase. Organization of the microtubule network is also influenced by changes in the nucleating activity of centrosomes in M-phase vs interphase. The nucleating activity of centrosomes seems to be under the control of M-phase specific kinases, since its modulation correlates with cell cycle-specific phosphorylation and dephosphorylation of certain centrosomal proteins (Vandre and Borisy, 1989; Vandre *et al.*, 1984; Kuang *et al.*, 1991).

In mouse oocytes arrested in M II, microtubules form almost only the meiotic spindle despite the fact that centrosomes are present not only at the spindle poles, but also in the cytoplasm as pericentriolar material (PCM) foci (Maro *et al.*, 1985). High Cc for tubulin polymerization in the oocytes prevents microtubule assembly at non-spindle PCMs, while the vicinity of chromosomes lowers this parameter enabling the formation of spindle microtubules (Karsenti *et al.*, 1984; Maro *et al.*, 1985). Lowering the overall Cc for tubulin polymerization by taxol induces the formation of microtubule asters around normally inactive cytoplasmic PCM foci (Maro *et al.*, 1985). These PCM foci become also active after oocyte activation or fertilization, and they are at the origin of the interphase network of microtubules (Maro *et al.*, 1985; Schatten *et al.*, 1985). The change of the PCM activity results probably from the lowering of the Cc for tubulin polymerization and from dephosphorylation of centrosomal proteins during the transition to the interphase. Mouse oocytes have only acentriolar centrosomes (Szöllösi *et al.*, 1972), and centrioles appear at the blastocyst stage (Maro *et al.*, 1991). Thus, the blastomeres of cleaving mouse embryos still lack centrioles, and the microtubule network is organized by PCM foci (Houliston *et al.*, 1987; Houliston and Maro, 1989).

Hybrid cells between mouse oocytes (arrested in M II or activated) and blastomeres (2-, 4-, and 8-cell embryos) described in this paper were made in order to follow the behavior of microtubules. After fusion, the interphase network of blastomere microtubules depolymerizes. This is followed by formation of a frame of new microtubules around the blastomere nuclei, which then transform into the spindle-like structures around prematurely condensed chromosomes in the M-phase arrested hybrids. A reciprocal influence of the interphase cytoplasm on oocyte metaphase microtubules leading to the formation of long microtubules in hybrids was observed when the participation of the interphase component was particularly increased. In activated hybrids, the blastomere nuclei underwent pronuclear-like transformation and the interphase network of microtubules formed.

## Results

During this study 133 hybrids between one or two blastomeres from 2-, 4- and 8-cell embryos and one M II oocyte or one oocyte undergoing activation around the time of fusion were analyzed. The blastomeres were used for fusion experiments while in the middle of the interphase to avoid the interference of their own progression into mitosis with fusion-related phenomena. None of the control blastomeres cleaved during the period of observations of the hybrids.

The interphase network of microtubules of the blastomeres (Fig. 1a,b) disappeared just after the fusion with either of the oocytes. In hybrids, where the oocyte component was in M II, the blastomere nuclei underwent PCC. The beginning of PCC was observed about 15-20 min after fusion with single 8-cell embryo blastomere, while it was significantly delayed in hybrids containing two 4-cell embryo blastomeres or two 2-cell embryo blastomeres and a single oocyte. In the first case the ratio of interphase cytoplasm to metaphase one was 1:8, while in the latter it was 1:2 and 1:1 respectively. When

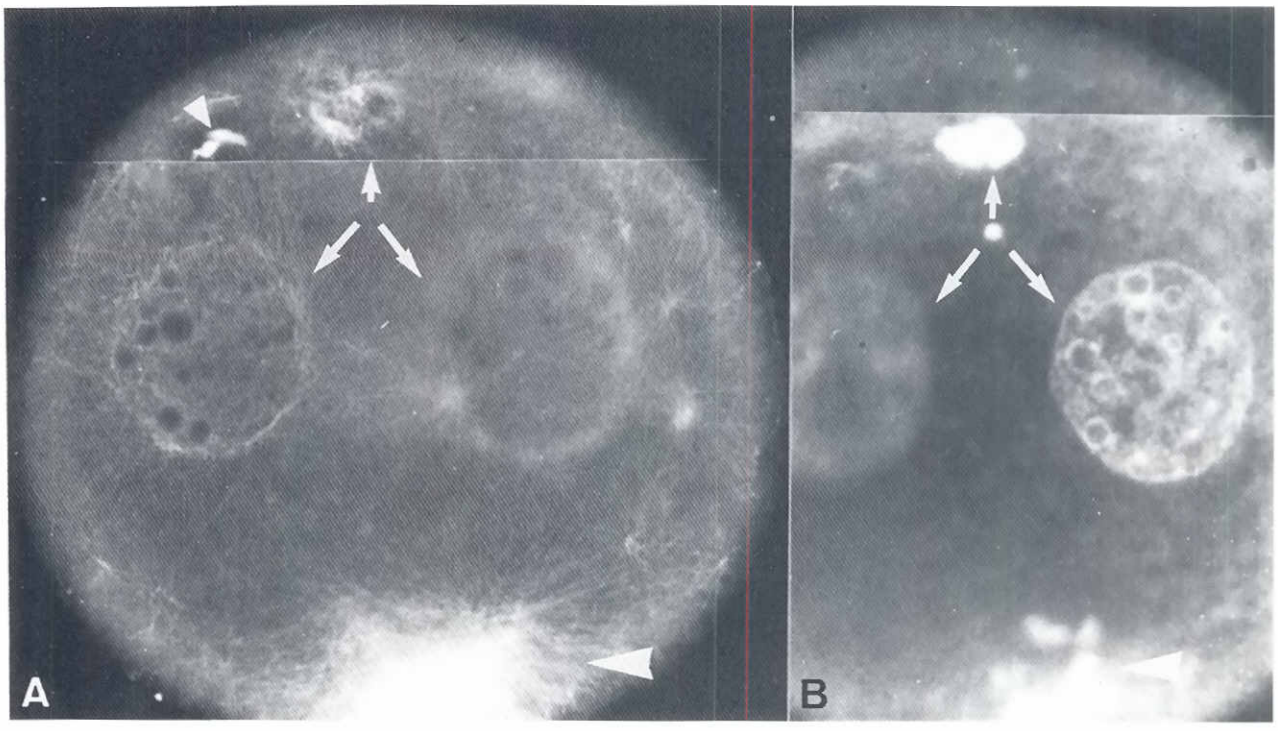


**Fig. 2. Hybrid of two 2-cell embryo blastomeres (whose nuclei are marked with large arrows) and their second polar body (small arrow) with non-activated oocyte 50 min after fusion.** *The ratio of the interphase, blastomere component to the metaphase, oocyte one in this hybrid is 1:1.* **(A)** *tubulin staining;* **(B)** *chromatin staining. Numerous microtubules appear around all nuclei introduced to the oocyte. The midbody of two sister blastomeres remains in the hybrid cell (small arrowhead). The meiotic spindle of the oocyte has numerous microtubules radiating around chromosomes (large arrowhead).*

two 2-cell embryo blastomeres were fused with a single oocyte their nuclei did not enter PCC during the first 50 min following fusion (Fig. 2b). The intact nuclei of the blastomeres became encircled with long microtubules radiating from the nuclear envelope towards the cytoplasm. Such microtubules formed also within the meiotic spindle, and numerous cytoplasmic asters of microtubules were observed in the hybrid cytoplasm (Fig. 2a). The microtubular frames around all kinds of blastomere nuclei became more evident when PCC began (Fig. 3a,b; 4a,b). In early hybrids made of one 8-cell embryo blastomere with one oocyte, only a few new microtubules appeared at the poles of the meiotic spindles (Fig. 4a). Later, however, these microtubules disappeared and the hybrids had unchanged meiotic spindles (Figs. 4c,e,g). These data demonstrate a significant facilitation of the polymerization of microtubules as the result of the introduction of interphase cytoplasm to the metaphase environment in the hybrids. Moreover, the degree and the duration of this burst of polymerization of microtubules increase when the volume of the introduced interphase cytoplasm increases.

When the condensing blastomere chromatin became irregular the microtubular frames collapsed and a few centers of accumulation of microtubules could be distinguished (Fig. 4c,d). The condensation of the chromosomes was accompanied by the formation of irregular spindle-like structures, which were often monopolar (Fig. 4 g,h), or tripolar (Fig. 4e,f) .

In hybrids undergoing activation around the time of fusion the extrusion of the second polar body was observed similarly to control activated oocytes. The network of interphase microtubules of the blastomeres disappeared and polymerization of new microtubules around the introduced nuclei was observed as in non-activated hybrids (data not shown). However, the blastomere nuclei did not enter PCC, but started to swell and undergo a pronuclear-like transformation. The introduced nucleus swelled faster than the pronucleus formed within the oocyte (Fig. 5b). Such hybrids developed an interphase network of microtubules similar to the one observed in control activated oocytes (Fig. 5a).

## Discussion

As expected, the fusion of two cells at different stages of the cell cycle (oocytes in M II and interphase blastomeres) triggered rearrangements of the microtubule network of the interphase component, but also in some cases, of the metaphase one. These rearrangements involved the rapid disassembly of blastomere interphase microtubules when they were in contact with the metaphase cytoplasm of the oocyte and the subsequent formation of new microtubules around the introduced nuclei. Further evolution depended clearly on the cell cycle conditions in which the egg component was at the moment of fusion: M II-arrested or activated oocytes. In M II oocytes the introduced chromatin induced the formation of numerous microtubules that transformed into spindle-like structures. In the activated oocytes the nucleus-associated frames of microtubules disappeared and were replaced by a

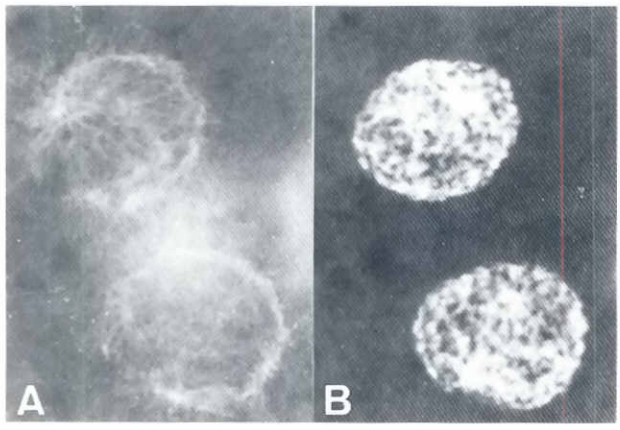


**Fig. 3. Two nuclei of 4-cell embryo blastomeres 50 min after fusion with non-activated oocyte.** *The ratio of the interphase component to the metaphase one in this hybrid was 1:2.* **(A)** *tubulin staining;* **(B)** *chromatin staining. The nuclei that started premature chromatin condensation* **(B)** *are encircled with more developed microtubule frames* **(A)** *than the still intact nuclei in the hybrid on Fig.2.*

cytoplasmic network. The initial reaction (disassembly of interphase microtubules and formation of nuclei-associated microtubules) was similar for activated and non-activated hybrids and resulted probably from a delay in the disappearance of the metaphasic conditions in the freshly activated ones (Szöllösi *et al.*, 1988). In other words, the blastomere nuclei introduced into the oocyte at the moment of activation were exposed to M-phase conditions, which progressively disappeared. It was demonstrated by Szöllösi *et al.* (1986b, 1988) by ultrastructural studies of similar hybrids made between mouse oocytes and thymocytes, that freshly activated oocytes are still capable (for up to 2 h after activation) to induce the initial changes typical for PCC, *i.e.*, partial condensation of chromatin and dissolution of the nuclear envelope of the introduced nuclei. Later, however, the nuclear envelope reforms, the chromatin decondenses and the blastomere nuclei undergo pronuclear-like transformation. It was also demonstrated that fully interphase conditions in activated oocytes are only established a few hours after activation. The nuclear lamins seem to persist in a soluble form in such oocytes for 3-5 h (Kubiak *et al.*, 1991b), and M-phase-like phosphorylation of certain proteins such as the 35 kDa complex and 46 kDa complex (Howlett and Bolton, 1985; Howlett, 1986) are observed also for a prolonged period after oocyte activation, in contrast to mitotic cell cycles of cleaving embryos, when their dephosphorylation is observed very shortly after completion of the cleavage division. Our observations confirm that the activation of mouse oocytes is followed by a period of metaphase-like cytoplasmic conditions, which promotes disassembly of interphase microtubules followed by the formation of nuclei-associated frames of microtubules.

The influence of the interphase cytoplasm on the behavior of microtubules in hybrids leads to a facilitated polymerization of microtubules in hybrids containing a significant volume of interphase cytoplasm (one M II oocyte and two 4- or 2-cell embryo blastomeres). It was demonstrated *in vitro* that the active cdc2 protein kinase inhibits microtubule growth, while its inactivation favors microtubule

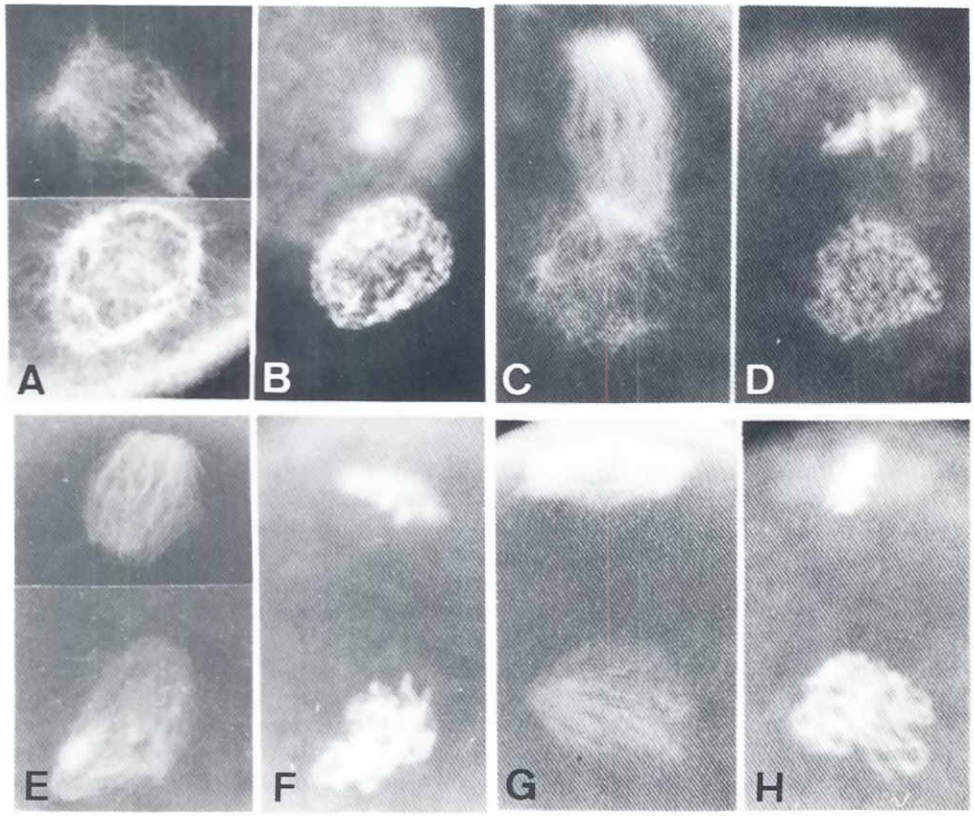


**Fig. 4. Microtubules accompanying prematurely condensing chromatin of 8-cell embryo blastomere introduced to non-activated oocyte. (A,C,E,G) tubulin staining; (B,D,F,H) chromatin staining.** *The meiotic spindles and chromosomes of the oocytes - in the upper part of each figure; the blastomere nuclei - in the lower part. Microtubules form dense frames around blastomere nuclei* **(A)** *when premature chromatin condensation starts 20 min after fusion* **(B)**. *After nuclear envelope breakdown the chromatin of the blastomere becomes irregular* **(D)** *and the microtubule frame collapses showing few distinct foci of microtubules 30 min after fusion* **(C)**. *Individual chromosomes become visible 50 min after fusion (F), while microtubules form a spindle-like structure* **(E)** *here tripolar. The spindle-like structures formed around chromosomes of the blastomere origin* **(H)** *are often irregular, like the monopolar structure 60 min after fusion (G). Note that the meiotic spindles in the early hybrid (20-30 min after fusion; A, C) have additional microtubules at the poles, which then disappear (50-60 min after fusion; E, G).*

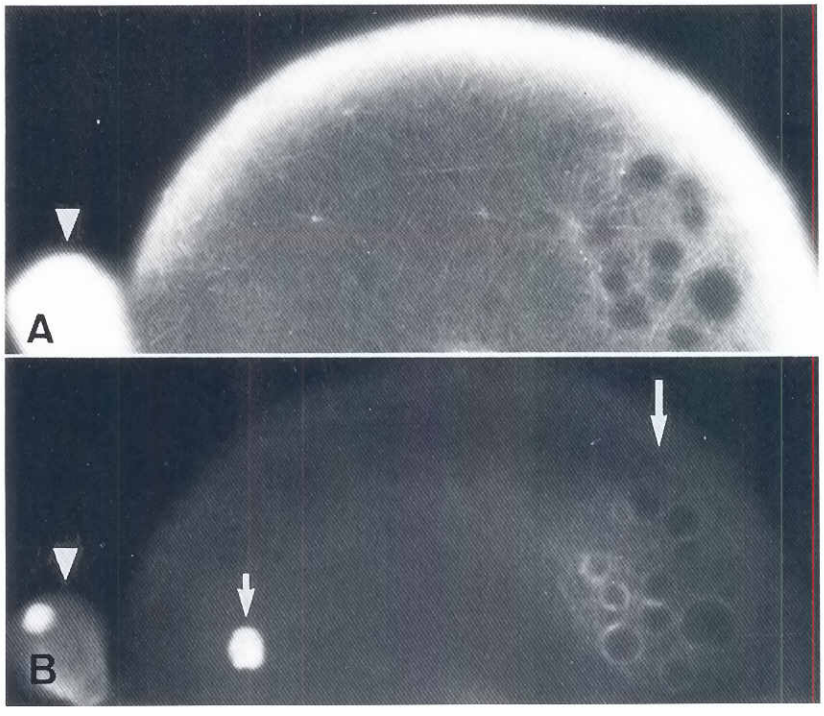


**Fig. 5. Hybrid of a single 8-cell embryo blastomere and activated oocyte 3 h after fusion. (A) tubulin staining; (B) chromatin staining.** *The oocyte completed meiosis after fusion and extruded the second polar body (arrowhead). The oocyte's own pronucleus is retarded in development (small arrow), while the blastomere nucleus (large arrow) undergoes pronucleus-like transformation* **(B)**. *Microtubules form a cytoplasmic network in the hybrid* **(A)** *similar to that observed in activated, unfused oocytes.*

elongation (Verde *et al.*, 1990). In hybrids, a similar effect is observed when the metaphase cytoplasm is "diluted" by introduction of a significant volume of blastomere interphase cytoplasm. However, this reaction does not persist for a prolonged period and if the hybrid remains in M-phase, the disassembly of newly formed cytoplasmic microtubules and the formation of chromatin-associated, spindle-like microtubules is observed. The initial burst of microtubule polymerization in these hybrids proceeds probably not only due to simple dilution, but also to a partial inactivation of the cdc2 kinase by certain cytoplasmic factors inhibiting this activity and potentially present in the interphase blastomeres. The presence of such factor(s) was demonstrated in early mouse zygotes (Balakier and Masui, 1986). These factors might include phosphatases, like the phosphatase 2A (Felix *et al.*, 1990; Jessus *et al.*, 1991). The opposite action of kinases and phosphatases is now well established as a mechanism governing phosphorylation/dephosphorylation of proteins, which in turn seems to be a major mechanism controlling the cell cycle transitions (ibid.). Our observations show a reciprocal action of metaphase and interphase cytoplasm on the behavior of microtubules in mouse oocyte-blastomere hybrids, and thus suggest a similar mechanism regulating microtubule growth as described for *Xenopus laevis* cytoplasm *in vitro* (Verde *et al.*, 1990; Karsenti *et al.*, 1991).

The sequence of spindle-like structure formation in M II-arrested hybrids demonstrates an important role of the chromatin and of the nuclear envelope in the nucleation and organization of prophase and pro-metaphase microtubules in the case of acentriolar cells. The nucleating action of the nuclear envelope was postulated in acentriolar plant cells (Lambert, 1980) as well as in acentriolar myogenic tubes (Tassin *et al.*, 1985). The sequence of events during spindle-like structure formation is similar to the one observed during the formation of the metaphase spindle in acentriolar plant cells of Heamanthus endosperm (De Mey *et al.*, 1982) and onion root (Kubiak *et al.*, 1986; Kubiak and Tarkowska, 1987) as well as during germinal vesicle breakdown and the formation of the first meiotic spindle in mouse oocytes (Szöllösi, 1976; Rime *et al.*, 1987; Kubiak *et al.*, 1989; Maro *et al.*, 1990; Van Blerkom, 1991). It seems that the formation of the spindle-like structures in hybrids involves the initial nucleation of microtubules around the nuclear envelope and the local decrease of the Cc for tubulin polymerization in the vicinity of the chromosomes.

The spindle-like structures in the hybrids show numerous abnormalities, and we found very often apolar, monopolar, or tripolar structures. It seems probable that during their formation PCM foci are recruited from the cytoplasm to form the poles of these structures, since their shape is more similar to the broad meiotic spindles than the more focused blastomere mitotic spindles (Schatten *et al.*, 1985). A random recruitment of these foci during PCC might result partially in their unorganized structures. Also, the kinetochores of prematurely condensed chromosomes seem to be incompetent to anchor microtubules properly (Szöllösi *et al.*, 1986b). Both these factors might interfere with the formation of normal spindles around the blastomere-derived chromosomes during premature chromatin condensation.

## Materials and Methods

### *Mice*

Randomly bred Swiss albino or F1(DBA/C3H) females were induced to ovulate by intraperitoneal injection of 10 IU PMSG and 42-48 h apart 5 IU HCG.

### *Oocytes*

Ovulated oocytes were released from ampullae into 0.1% hyaluronidase (Sigma) in M2 medium (Fulton and Whittingham, 1978) 14-15 h after HCG injection.

***Embryos***

Cleaving embryos were obtained from Swiss albino or F1(DBA/C3H) females, which had mated after HCG injection with F1(DBA/C3H) males. 2-, 4- and 8-cell embryos were flushed from the oviducts 36-40, 58-60 and 68-70 h after HCG injection, respectively. Phosphate-buffered saline (PBS) was used for recovery of embryos. Zonae pellucidae were removed enzymatically by brief digestion with 0.5% pronase (Sigma). Embryos were disaggregated into single blastomeres in calcium and magnesium-free PBS (Dulbecco) by gentle pipetting at room temperature.

***Agglutination and electrofusion***

Oocytes were agglutinated with blastomeres in phytohemagglutinin (PHA; Wellcome) diluted in M2 without BSA in final concentration of 150 μg/ml for 5 min on 1.2% agarose at room temperature. Aggregated pairs were washed in 0.25 M glucose made in bidistilled water and electrofused in this solution according to the technique described previously (Kubiak and Tarkowski, 1985). Two direct current pulses of 17 V and 140 μsec duration were applied from a pulse generator PM 5715 (Philips). The pairs were transferred to drops of M2+BSA under liquid paraffin at 37°C. They were scored every 5-10 min and fixed every 10-15 min during the first hour after fusion and 1 h 30 min post-fusion.

***Fixation***

Hybrid cells and control, unfused pairs were stuck to coverslips coated with 1 mg/ml poly-L-lysine as described by Mazia *et al.* (1975), or with 150 μg/ml PHA for 5-8 min and fixed in 0.5% glutaraldehyde with 0.05% Triton X-100 in PBS for 10 min. Then they were washed in PBS, permeabilized with 0.5% Triton X-100 for 30 min, washed again in PBS and incubated in 2 mg/ml $NaBH_4$ in PBS for 15 min.

***Immunofluorescence and nuclear staining***

The specimens were incubated in normal goat serum diluted 1/20 in 0.01 M Tris buffer (TBS) for 30 min, then overnight in rabbit anti-dog brain tubulin antibody diluted 1/50 in 1% normal goat serum (final concentration 50 μg IgG/ml). The cells were washed with TBS, incubated for 1 h in GAR/FITC (Nordic Imm.) diluted 1/40 in TBS and washed with PBS. The specimens were then incubated in 30 μg/ml propidium iodide in PBS, washed in PBS and mounted in Gelvatol supplemented with DABCO (Langanger *et al.*, 1983). The cells were observed under a Nikon fluorescence microscope and photographed on TX-400 Kodak film.

*Acknowledgments*

*I would like to thank Marc De Brabander and Jan De Mey for a kind invitation to work in their laboratory (Janssen Pharmaceutica Res. Lab., Beerse, Belgium), where this study was done, to Evelyn Houliston, Bernard Maro, Gerald Schatten and Dan Szöllösi for valuable discussions and critical reading of the manuscript and Richard Schwartzmann for excellent photographic work.*

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*Accepted for publication: September 1991*