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Mitotic Factors From Mammalian Cells: A Preliminary Characterization

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The objective of this study was the preliminary characterization of the factors from mitotic HeLa cells that can induce meiotic maturation in Xenopus laevis oocytes. We found that this factor is a heat-labile, Ca^{2+}-sensitive, nondialyzable protein with a sedimentation value of 4-5S. Furthermore, no new protein synthesis was found to be required for this mitotic factor to induce maturation in the amphibian oocytes. These data suggest that the factors involved in the breakdown of nuclear membrane and the condensation of chromosomes that are associated with three different phenomena, mitosis, meiosis, and premature chromosome condensation, are very similar in different animal species.

Key words: mitotic factors, oocyte maturation

In amphibians, meiotic maturation of ovarian oocytes involves the breakdown of the germinal vesicle (nucleus), chromosome condensation, and progression through the first meiotic division. This maturation process can be induced either by incubating fully grown oocytes with progesterone or by normal ovulation in vivo [1–5]. Since maturation is not induced when progesterone is injected into the oocyte [6, 7], the existence of a cytoplasmic factor, produced in the oocyte in response to progesterone treatment, has been postulated. Indeed, when cytoplasmic extracts from matured oocytes are injected into immature oocytes they induce maturation [7–9]. Recent reports suggest that maturation-promoting activity (MPA) is present not only in maturing oocytes but also in early cleavage stages of amphibian embryos undergoing synchronous cell division [10]. The MPA appears to fluctuate during the division cycle of the embryonic cells and reaches a peak during mitosis [10]. The factor from the mature oocytes was found to be a heat-labile protein that is magnesium-dependent and calcium-sensitive and exists in three different molecular sizes [9].

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Recently we have shown that extracts of mitotic HeLa cells can induce germinal vesicle breakdown (GVBD) and chromosome condensation in amphibian oocytes, indicating that these factors have no species barriers [11]. Further, we observed a cyclical change in the levels of MPA during the HeLa cell cycle. The MPA was not present in either G_1 or S phase cells. The mitotic factors accumulated slowly in the beginning of G_2 but proceeded at a progressively rapid rate during late G_2 and reached a threshold at the G_2-mitotic transition when the nuclear membrane breaks down and chromatin condenses into chromosomes. The present study is an attempt at preliminary characterization of the mitotic factors from HeLa cells that induce maturation in amphibian oocytes.

MATERIALS AND METHODS

Cells and Cell Synchrony

HeLa cells were grown as spinner cultures at 37°C in Eagle's minimal essential medium (MEM) supplemented with nonessential amino acids, heat-inactivated fetal calf serum (10%), sodium pyruvate, glutamine, and penicillin-streptomycin mixture [12]. These cells have a cell cycle time of 22 hours consisting of 10.5 hr of pre-DNA synthetic (G_1) period, 7 hours of of DNA synthetic period, 3.5 hr of post-DNA synthetic (G_2) period and 1.0 hour of mitosis. To obtain mitotic populations, HeLa cells were first partially synchronized by a single TdR block, and then they were incubated at 37°C for about 10 hr in a chamber filled with N_2O at a pressure of 80 lb/in^2 (5.36 atm). The rounded and loosely attached mitotic cells were selectively detached by gentle pipetting, which yielded a population with a mitotic index of 98% [13].

Preparation of HeLa Cell Extracts

Extracts of mitotic cells were prepared by suspending cells at a concentration of 20 $\times$ 10^6 cells/ml of extraction medium containing 0.2M NaCl, 0.25M sucrose, 0.01M $MgSO_4$, 0.002M EGTA, and 0.01M Na_2HPO_4/NaH_2PO_4, pH 6.5 [9, 11]. In some experiments Ca^{2+}, Mg^{2+}, and EGTA-free buffer was used. Cell extracts were obtained by hand homogenization using a Teflon pestle/glass homogenizer (20 strokes) at 4°C. The homogenate was centrifuged at 30,000 $\times$ g for 30 min in a refrigerated Sorvall RC5 centrifuge at 4°C. The supernatants thus obtained were used for the assay of MPA in Xenopus laevis oocytes. Protein concentration in the extracts was determined by the method of Bradford [14] using bovine serum albumin as the standard.

Preparation of Xenopus Oocytes

Oocytes were obtained by surgically removing a portion of the ovaries from X. laevis females. With a small incision that can be closed by a few stitches, multiple harvests of oocytes can be obtained from the same animal. All operations on oocytes were conducted using amphibian Ringer's solution supplemented with $MgCl_2 \cdot 6H_2O$ (0.12 grams/liter). Oocytes were manually dissected from their follicles after pretreatment of the ovarian fragments with collagenase (1 mg/ml) [3].

Assay for Maturation Promoting Activity

Cell extracts were assayed by injecting 65 nl into each oocyte. Injected oocytes were inspected for GVBD after 1 to 3 hr. Germinal vesicle breakdown was detected by a depigmentation of an area of the animal hemisphere [3]. Presence or absence of the germinal vesicle was also determined by dissection of the oocyte. Some oocytes were fixed in Smith's fluid, dehydrated with amylacetate [15], embedded in paraffin, sectioned at 7 μ and stained with Feulgen and fast green [16].

Sucrose Gradient Density Centrifugation

5-ml linear 5–20% sucrose gradients in 10 mM phosphate buffer (pH 6.5) containing 0.002M EGTA were prepared in polyallomer centrifuge tubes. The 100,000 × g supernatants obtained from mitotic cell extracts (0.4 ml) were layered on the gradients and centrifuged at 85,000 × g for 17 hr in a Beckman SW 50.1 rotor at 4°C. The gradients were fractionated into 0.25-ml aliquots using an ISCO microfractionater. Each fraction was used to assay for MPA and protein content. These fractions were also assayed for the presence of mannose phosphate isomerase, malate dehydrogenase, 6 phosphogluconate dehydrogenase, lactate dehydrogenase, and malic enzyme [17], present in HeLa cell extracts, as internal standards for determining the sedimentation profile.

RESULTS

Throughout this study either whole or subfractions of the mitotic extracts were assayed for their MPA as indicated by GVBD and chromosome condensation in Xenopus oocytes. The results of this study indicate that maturation could be induced in 100% of the oocytes injected with the mitotic extracts. In some of the oocytes we could clearly see the first polar body (Fig. 1).

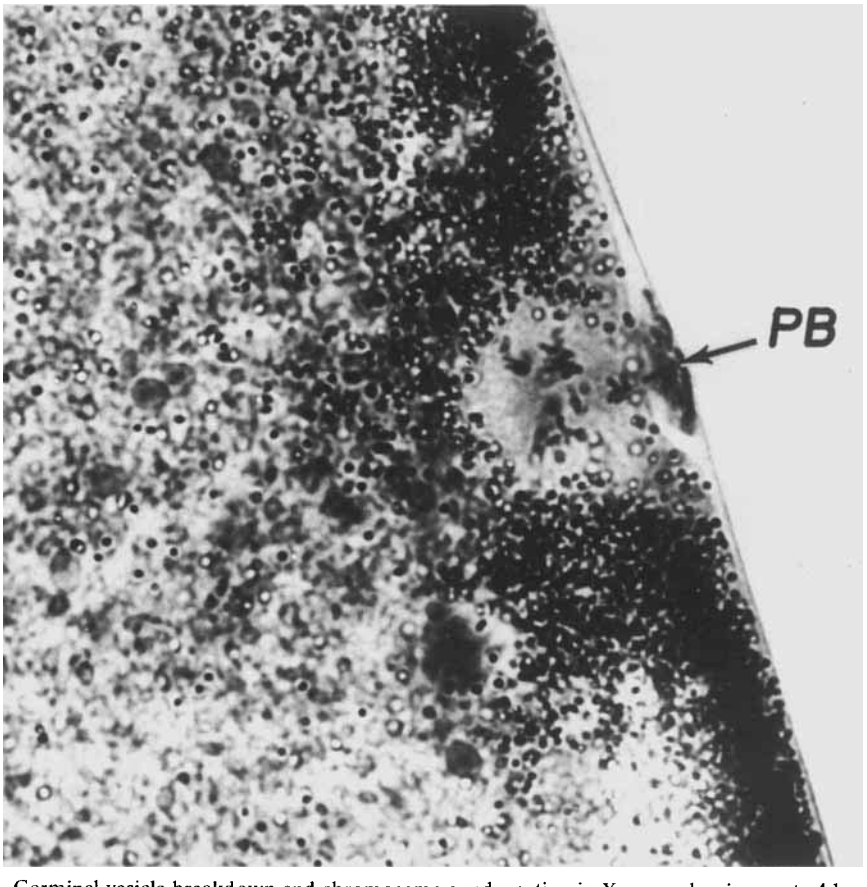

Fig. 1. Germinal vesicle breakdown and chromosome condensation in Xenopus laevis oocyte 4 hr after injection with HeLa cell mitotic extract (65 nl containing a total of 221 ng protein). Oocyte fixed in Smiths fluid; sections stained with Feulgen-fast green. Magnification approximately 1,320×. Note the presence of first polar body (PB) between plasma membrane of the egg and the vitelline membrane. The condensed chromosomes of the oocyte are located on the spindle of the arrested second meiotic division in the region relatively clear of pigment granules.

Nature of Mitotic Factors

Incubation of freshly prepared mitotic extracts (7 mg/ml) with RNase (1.5 units/ml) at 25°C for 1 hr had no effect on the MPA (Table I). However, a similar treatment with protease (subtilopeptidase-A from B. subtilis)(0.2 units/ml) reduced this activity to 18% as compared to 100% in untreated extract. Neither RNase nor protease at the concentrations tested showed any toxic effects on the oocytes. These enzymes per se were unable to induce GVBD.

Effect of Cycloheximide on Maturation Promoting Activity of Mitotic Factors

Oocytes were incubated for 1 hr in Ringer's solution containing cycloheximide at a concentration (20 μg/ml) known to inhibit protein synthesis in amphibian oocytes [18]. These oocytes were then injected with mitotic extracts (7 mg/ml) and further incubated for 2 hr with cycloheximide (20 μg/ml). The data shown in Table II indicate that the MPA induced by the mitotic factors is not dependent on new protein synthesis.

Effect of Ca^{2+} and Mg^{2+} on Maturation Promoting Activity of the Mitotic Factors

The MPA of the mitotic factors, which was highly sensitive to Ca^{2+} (1 mM), was unaffected by Mg^{2+} even at relatively high concentrations (10 mM) (Table III).

TABLE I. Effect of RNase and Protease Treatments on the Maturation Promoting Activity of Mitotic Cell Extracts

Substance injected	No. of oocytes injected	No. showing GVBD	% GVBD
Mitotic extracts	11	11	100
RNase (1.5 units/ml) alone	11	0	0
Mitotic extracts treated with RNase (1.5 units/ml)	5	5	100
Protease (0.2 units/ml) alone	11	0	0
Mitotic extracts treated with protease (0.2 units/ml)	11	2	18

Freshly prepared mitotic extracts (7 mg of protein/ml) were incubated with either RNase or protease at 25°C for 1 hr. At the end of incubation, 65 nl of the treated extracts were injected into each oocyte.

TABLE II. Maturation Promoting Activity of Mitotic Cell Extracts in Cycloheximide-Treated Oocytes

Treatment	No. of oocytes	No. showing GVBD	% GVBD
Injected with mitotic extract	11	11	100
Incubated with cycloheximide (20 μg/ml) alone	8	0	0
Mitotic extracts injected into cycloheximide-treated oocytes	12	12	100

Oocytes were incubated with cycloheximide (20 μg/ml) for 1 hr prior to injection. After injection of the mitotic extract, oocytes were further inucbated with cycloheximide for another 1.75 hr at which time they were scored for GVBD.

Heat-Sensitivity of the Mitotic Factors

Incubation of the mitotic extract at 40°C for 10 min had no significant effect on its MPA (Table IV). However, a 10-min incubation at 50°C or higher resulted in a rapid loss of this activity as evidenced by the decrease in the frequency of GVBD. Extracts incubated at 60° and 100°C did show some precipitate upon centrifugation. On the other hand heat treatment of the extract at 50°C did not give any precipitate upon centrifugation but showed a rapid loss of activity ruling out the possibility of mitotic factor being entrapped in the precipitated material.

Effect of Dialysis and pH of the Extraction Medium on the Activity of the Mitotic Factors

The MPA of the mitotic extracts was not affected by dialysing it against extraction medium of different pH's (Table V). However, the activity of the extract was lost when dialized against extraction medium with a pH of 8.0.

TABLE III. Effect of Ca^{2+} and Mg^{2+} on Maturation Promoting Activity of Mitotic Cell Extracts

Oocytes injected with	No. of oocytes injected	No. of oocytes showing GVBD	% GVBD
Ca^{2+} and Mg^{2+} - free buffer (CMF buffer)	7	0	0
Mitotic extract in CMF buffer	9	8	89
Mitotic extract in CMF buffer +1 mM Mg^{2+}	8	7	88
Mitotic extract in CMF buffer +2.5 mM Mg^{2+}	8	8	100
Mitotic extract in CMF +10 mM Mg^{2+}	13	13	100
Mitotic extract in CMF buffer +1 mM Ca^{2+}	10	0	0

A total volume of 65 nl of extracts made from 20×10^6 cells/ml (7 mg of protein/ml) was injected into each oocyte.

TABLE IV. Effect of Temperature on the Maturation Promoting Activity of Mitotic Cell Extracts

Temperature	No. of oocytes injected	No. of oocytes showing GVBD	% GVBD
0°C	10	9	90
40°C	12	10	83
50°C	12	1	8.3
60°C	6	0	0
100°C	6	0	0

Freshly prepared mitotic extracts were incubated at different temperatures for 10 min. At the end of incubation, extracts were centrifuged at 30,000 × g at 4°C to remove the precipitate. A total volume of 65 nl of the supernatant was injected into each oocyte.

Preliminary Characterization of the Mitotic Factors

In order to determine the molecular size of the factors that possess the MPA, the mitotic extract was centrifuged on linear sucrose density gradients (5–20%) and the various fractions collected were assayed for activity. Activity was observed in a single distinct peak (Fig. 2). The sedimentation value of this fraction was estimated to be about 4-5S.

TABLE V. Effect of pH of the Extraction Medium on the Maturation Promoting Activity of Mitotic Cell Extracts

pH of dialysis buffer	No. of oocytes injected	No. showing GVBD	% GVBD
Undialyzed	10	10	100
6.5	10	10	100
7.0	10	10	100
7.5	10	10	100
8.0	10	1	10

Mitotic extracts were dialyzed overnight against buffers of different pH and 65 nl of the dialyzed extracts were injected into each oocyte. The oocytes were examined for GVBD at 1.75 hr after the injection.

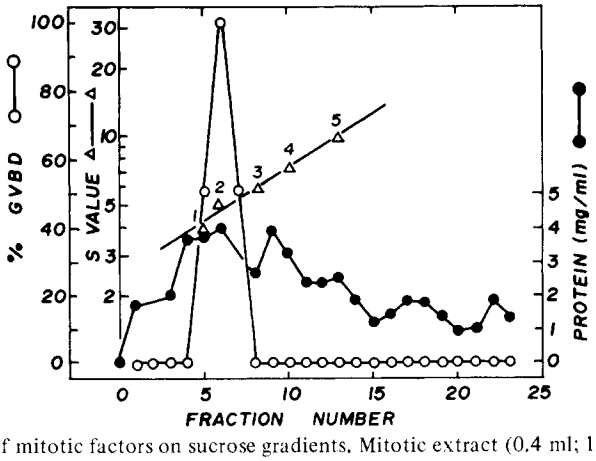

Fig. 2. Separation of mitotic factors on sucrose gradients. Mitotic extract (0.4 ml; 15 mg/ml) was layered on 5 ml linear sucrose gradient (5–20%) and spun at 85,000 × g for 17 hr in a Beckman SW50-1 rotor. The fractions were collected from the top by a fraction collector. Each of these fractions was assayed for maturation promoting activity and for the presence of (1) mannose phosphate isomerase (3.9S), (2) malate dehydrogenase (5S), (3) 6 phosphogluconate dehydrogenase (6S), (4) lactate dehydrogenase (7.3S), and (5) malic enzyme (10S) as internal standards.

DISCUSSION

The results of this study suggest that the factors from mitotic HeLa cells that can induce GVBD and chromosome condensation in Xenopus oocytes are heat-labile, nondialyzable and Ca^{2+}-sensitive proteins. It was also found that synthesis of new proteins was not required for the induction of maturation by the mitotic factors. Furthermore, the MPA appears to reside in a protein of a single molecular size of 4-5S.

The characteristics of the mitotic factors from HeLa cells appear to be similar to those of the maturation promoting factors isolated from the cytoplasms of mature amphibian oocytes, which were also found to be heat-labile and Ca^{2+}-sensitive proteins [9]. Unlike the mitotic factor that has a single molecular size (4-5S), the factor from the oocytes have three different forms, 4S, 15S and 32S [9].

The present study further confirms our earlier observation [11] that factors involved in the breakdown of the nuclear membrane and the condensation of chromosomes, which are associated with three different phenomena, mitotis, meiosis, and premature chromosome condensation, appear to be very similar if not identical in different animal species. Whether this particular factor (4-5S protein) from the mitotic cells, which induces maturation in amphibian oocytes, can also induce premature chromosome condensation in mammalian cells remains to be tested.

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REFERENCES

1. Masui Y: J Exp Zool 166:365, 1967.
2. Subtelny S, Smith LD, Ecker RE: J Exp Zool 168:39, 1968.
3. Merriam RW: J Exp Zool 180:421, 1972.
4. Wasserman WJ, Masui Y: Biol Reprod 11:133, 1974.
5. Dettlaff T, Nikitina LA, Stoeva OG: J Embryol Exp Morphol 12:851, 1964.
6. Masui Y, Markert CL: J Exp Zool 177:129, 1971.
7. Smith LD, Ecker RE: Dev Biol 25:233, 1971.
8. Drury KC, Schorderet-Slatkine S: Cell 4:269, 1975.
9. Wasserman WJ, Masui Y: Science 191:1266, 1976.
10. Wasserman WJ, Smith LD: J Cell Biol R 15, 1978.
11. Sunkara PS, Wright DA, Rao PN: Proc Natl Acad Sci USA 76:2799, 1979.
12. Rao PN, Engelberg J: Science 148:1092, 1965.
13. Rao PN: Science 160:774, 1968.
14. Bradford MM: Ann Biochem 72:248, 1976.
15. Drury HF: Stain Technol 16:21, 1941.
16. Subtelny S, Bradt C: J Morphol 112:45, 1963.
17. Sicilaino MJ, Shaw CR: In Smith I (ed): "Chromatographic and Electrophoretic Techniques." London: Heinemann, Volar, pp 185–209, 1976.
18. Wasserman W, Masui Y: Exp Cell Res 91:381, 1975.