Evidence for the Presence of Inhibitors of Mitotic Factors during G_1 Period in Mammalian Cells

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ABSTRACT Our earlier studies indicated that the mitotic factors, which induce germinal vesicle breakdown and chromosome condensation when injected into fully grown *Xenopus* oocytes, are preferentially associated with metaphase chromosomes and that they bind to chromatin as soon as they are synthesized during the G_2 phase. In this study, we attempted to determine the fate of these factors as the cell completes mitosis and enters G_1. Extracts from HeLa cells at different points during G_1, S, and G_2 periods were mixed with mitotic extracts in various proportions, incubated, and then injected into *Xenopus* oocytes to determine their maturation-promoting activity. The maturation-promoting activity of the mitotic extracts was neutralized by extracts of G_1 cells during all stages of G_1 but not by those of late S and G_2 phase cells. Extracts of quiescent (G_0) human diploid fibroblasts exhibited very little inhibitory activity. However, UV irradiation of G_0 cells, which is known to cause decondensation of chromatin, significantly enhanced the inhibitory activity of extracts of these cells. These factors are termed inhibitors of mitotic factors (IMF). They seem to be activated, rather than newly synthesized, as the cell enters telophase when chromosomes begin to decondense. The IMF are nondialyzable, nonhistone proteins with a molecular weight of >12,000. Since mitotic factors are known to induce chromosome condensation, it is possible that IMF, which are antagonistic to mitotic factors, may serve the reverse function of the mitotic factors, i.e., regulation of chromosome decondensation.

Chromatin undergoes profound structural alterations during the cell cycle. During mitosis, it is supercoiled and condensed to form the mitotic chromosomes. Chromosome condensation is a critical event in the cell cycle that is necessary for the equal distribution of genetic material between the daughter cells. The phenomenon of premature chromosome condensation described by Johnson and Rao (13) has been very useful in visualizing the state of chromatin condensation during interphase. Using this technique, Rao and Hanks (30) and Hanks and Rao (9) have shown that the process of chromosome decondensation, initiated during the telophase of mitosis, continues throughout the G_1 period until the chromatin reaches its ultimate state of decondensation by the end of G_1, when DNA synthesis is initiated.

As for the factors involved in chromosome condensation, studies from this and other laboratories have demonstrated that when extracts from mitotic HeLa cells (39, 40) or mitotic Chinese hamster ovary cells (19) are injected into *Xenopus laevis* oocytes, they exhibit maturation-promoting activity (MPA)[1] as evidenced by germinal vesicle breakdown (GVBD) and condensation of chromosomes. These studies also revealed that the mitotic factors are nonhistone proteins and accumulate slowly in the beginning of G_2, increase rapidly during late G_2, and reach a threshold at the G_2-mitotic transition when the chromatin is transformed into discrete chromosomes (34, 38). The mitotic factors have great affinity for chromatin and preferentially bind to it as soon as they are synthesized in G_2 phase; as the cell synthesizes more of these factors in preparation for mitosis, increasing amounts of them are retained in the cytoplasm (1). These chromosome-bound mitotic factors can be released by mild digestion with endonucleases (2).

[1] *Abbreviations used in this paper:* GVBD, germinal vesicle breakdown; IMF, inhibitors of mitotic factors; MPA, maturation-promoting activity.

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The objective of the present study was to investigate the fate of mitotic factors as the cell divides and chromosomes begin to decondense at telophase. Do the mitotic factors that associate with chromatin during G_2 and mitosis dissociate, or are these factors inactivated in situ by some other factors newly synthesized or activated as the cell divides? The results of this study indicate the existence of certain factors (proteins) during the G_1 period of mammalian cells that specifically inactivate the mitotic factors as indicated by the loss of their MPA when injected into oocytes. An abstract of this study has appeared elsewhere (27).

MATERIALS AND METHODS

Chemicals

^{3}H-L-amino acid mixture and ^{14}C-L-amino acid mixture with identical L-amino acid activities were obtained from New England Nuclear (Boston, MA). 2-Mercaptoethanol and Coomassie Brilliant Blue G-250 protein-binding dye were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of reagent quality and were mainly purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cells and Cell Synchrony

HeLa cells: HeLa cells were routinely grown as suspension cultures in spinner flasks at 37°C in Eagle's minimum essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (K C Biologicals, Inc., Lenexa, KS), sodium pyruvate, nonessential amino acids, and penicillin-streptomycin mixture (Gibco Laboratories) in a humidified 5% CO_2 atmosphere, as described earlier (28). These cells have a cell cycle time of 22 h, consisting of a 10.5-h G_1 period, a 7.0-h S period, a 3.5-h G_2 period, and a 1-h mitosis (29).

To obtain mitotic cells, HeLa cells in exponential growth were plated in 1,585 cm^2 borosilicate roller bottles (Bellco Glass Co., Vineland, NJ) as recently described (2). Briefly, cells were partially synchronized into S phase by a single excess thymidine (2.5 mM) block of 20 h. After reversal of the thymidine block by washing, cells were incubated in fresh medium containing Colcemid (0.05 μg/ml) (Ciba Pharmaceutical Co., Summit, NJ) for another 20 h at 37°C, and mitotic cells were harvested by selective detachment.

To obtain a pure population of HeLa cells in S and G_2 phases, we first synchronized exponentially growing cells into S phase by the excess thymidine double-block method (5, 29). Early-, mid-, and late-G_2 cells were obtained by harvesting cells at 8, 9, and 10 h after reversal of the second thymidine block and incubation with Colcemid. Colcemid-arrested mitotic cells were removed by selective detachment and discarded. The G_2 cells that remained attached to the surface were trypsinized and used for the experiments. The mitotic indices in all these populations were never >3%.

HeLa cells synchronized in various stages of G_1 phase were obtained by reversing the nitrous oxide-blocked mitotic cells as described previously (26). Within 1.5 h after reversal of N_2O block, >90% of the cells had completed mitosis and entered G_1.

WI-38 cells: A normal human diploid fibroblast cell line, WI-38, was obtained from the American Type Culture Collection, Rockville, MD. These cells were grown as monolayers in Lux plastic culture dishes in McCoy's 5A medium (Gibco Laboratories) with 10% heat-inactivated fetal calf serum supplemented with glutamine and penicillin-streptomycin mixture in a humidified CO_2 (5%) incubator at 37°C. G_0 cells were obtained by harvesting cells 7 d after they had reached confluency.

UV Irradiation of G_0 Cells

The WI-38 G_0 cells were washed thoroughly with PBS (pH 7.2) before a 10-s irradiation with a germicidal lamp emitting 90 ergs cm^{-2} sec^{-1} at 254 nm measured at the position of the cells. After UV irradiation, cells were incubated in fresh medium in the presence or absence of DNA synthesis inhibitors hydroxyurea (10^{-2} M) or arabinosylcytosine (10^{-4} M) for various times at 37°C before being harvested. In experiments designed to determine if UV irradiation induced new protein synthesis or activated existing proteins, cycloheximide (25 μg/ml) was added at the time of irradiation and kept in the medium during posttreatment incubation.

Preparation of Cytoplasmic, Nuclear, and Chromosomal Extracts

Cells synchronized in various phases were collected at 4°C by centrifugation at 1,000 rpm for 5 min. After three washings with Eagle's minimum essential medium without serum at 4°C, the cytoplasmic, nuclear, or chromosomal extracts were prepared as described previously (1, 2). Briefly, cells were lysed in a modification of the buffer of Blumenthal et al. (4), buffer A: 15 mM Tris-HCl, pH 7.4, containing 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 15 mM 2-mercaptoethanol, 2 mM EDTA, 0.5 mM EGTA, supplemented with the protease inhibitor 1 mM phenylmethylsulfonyl fluoride, the phosphatase inhibitors 1 mM ATP, 5 mM NaF, and 5 mM sodium β-glycerolphosphate, and 0.25 M sucrose. After centrifugation the chromosomal or nuclear pellet was washed once again with the same buffer and then resuspended in the high-salt buffer B: 10 mM Na_2HPO_4/NaH_2PO_4, 200 mM NaCl, 2 mM EGTA, 10 mM $MgSO_4$, 1 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 5 mM sodium β-glycerolphosphate and 0.25 M sucrose at pH 6.5, to extract a subset of nonhistone chromatin-binding proteins. The cytoplasmic, nuclear, or chromosomal extracts were further centrifuged at 100,000 *g* for 1 h in a TI 50 rotor in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The pellets were discarded and aliquots from the resulting clear supernatants were injected into *Xenopus laevis* oocytes to determine their MPA. For some whole-cell extracts, cells were suspended in buffer B and lysed by sonication (3 × 10 s) and then centrifuged at 100,000 *g*. The extracts were always stored at −70°C until further use.

Preparation of Xenopus Laevis Oocytes and Assay for Maturation Promoting Activity

All the procedures employed for the preparation of oocytes and assay of the cell extracts for MPA by microinjection into oocytes were essentially the same as described earlier (1, 2). The presence or absence of the germinal vesicle for the questionable oocytes was also determined by fixation in 7.5% trichloroacetic acid and subsequent dissection of the oocyte before scoring for GVBD induction. For every set of oocytes removed from *Xenopus*, a batch was always tested for normal maturation by stimulation with progesterone (10 μg/ml) for 15 min and incubated for ~8 h before scoring for GVBD induction. As a further control, for every series of injections with extracts, about 10 oocytes were injected with extraction buffer.

Determination of the Protein Content

The protein concentration was determined by the use of a Coomassie Brilliant Blue G-250 dye-binding assay according to Bradford (6), using BSA as the standard.

Treatment of G_1 Cell Extracts

All treatments to study the effect of proteases, RNase, chelating agents, temperature, and pH on G_1 factors were performed on freshly prepared early-G_1 cell extracts before they were mixed with mitotic extracts in various proportions and eventually injected into oocytes to determine their MPA. Control experiments were simultaneously performed on mitotic extract diluted with extraction buffer.

RESULTS

The Fate of Mitotic Factors during Mitosis to G_1 Transition

To determine whether G_1 cells contained factors that could inactivate the mitotic factors at the end of mitosis, mitotic cell extracts were mixed with G_1 cell extracts in different proportions and the mixtures were injected into oocytes to test for MPA (Fig. 1). Mitotic cell extracts diluted with extraction buffer in corresponding proportions served as controls. It is evident from Fig. 1 that the mitotic cell extract could be diluted with the extraction buffer down to a 20% concentration without any significant loss of MPA. However, when the mitotic cell extract was mixed with the G_1 cell extract, we noticed a rapid inactivation of the mitotic factors even at mitotic extract concentration as high as 66.6%. In

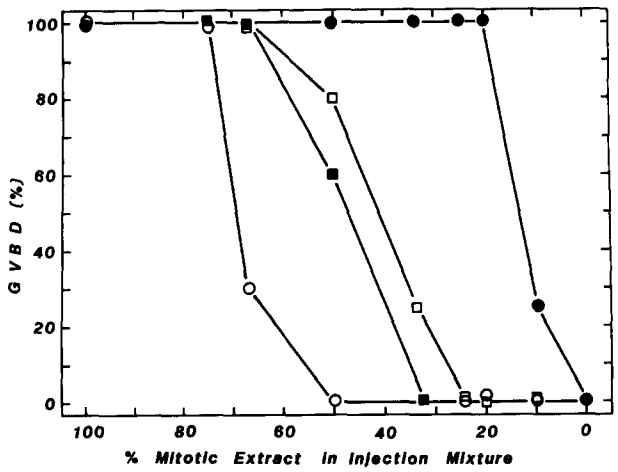

FIGURE 1 Effect of G_1 cell extracts on the MPA of the mitotic cell extracts. HeLa cells in G_1 phase were collected at different times (i.e., at 1.5, 2, 3, 4, 5, 6, 7, and 7.5 h) after reversal of the N_2O block. G_1 cell extracts were made using 6–8 × 10^7 cells/ml with a protein content ranging from 8–11 mg/ml. Similarly, mitotic cell extracts were made using 4 × 10^7 cells/ml containing ~8 mg/ml of protein. Whenever necessary, extracts were concentrated to give a protein content of 8 mg/ml by ultrafiltration using Amicon YM-10 filters. Extracts of G_1 cells at different points during G_1 were separately mixed with mitotic extracts in various proportions so as to obtain a mitotic extract concentration of 100, 75, 66.6, 50, 33.3, 25, 20, 10, and 0% in the injection mixture. These mixtures were incubated for 1 h at 4°C prior to injection into *Xenopus* oocytes. For each dilution a minimum of 10 oocytes was injected. A volume of 65 nl (containing ~50–550 ng of proteins) was usually injected. The percentage of GVBD in the injected oocytes was determined at 2–3 h after injection by scoring the oocytes for the appearance of a white spot in the animal hemisphere. In doubtful cases oocytes were fixed in 7.5% trichloroacetic acid and dissected to check for the breakdown of the germinal vesicle. Mitotic cell extracts diluted with the extraction buffer (in the presence or absence 10 mg/ml of bovine serum albumin) to give corresponding concentrations served as controls. These data represent an average of five experiments and the differences in % GVBD between the experiments did not exceed 10%. Typically oocytes from the same female were used for a given experiment. Dilution of mitotic extract with extracts from G_1 cells at 1.5 h, □, 2 h, ○, and 7.5 h, ■, after reversal of the N_2O block. Dilution with buffer, ●. The data obtained with extracts from G_1 cells collected at 3, 4, 5, 6, or 7 h after reversal were identical to those of G_1 cells at 2 h, and hence these data are not presented.

general, when mitotic and G_1 cell extracts with equal protein content were mixed, a complete inactivation of the mitotic cell extract occurred as its concentration was reduced to 50%. These experiments were repeated several times with highly reproducible results. As another control we added 10 mg/ml of BSA as a carrier protein to the extraction buffer before mixing with the mitotic extracts and observed no inactivation of the mitotic factors even at concentrations as low as 20%, as in the case of regular buffer control.

Extracts from HeLa G_1 cells collected at any time from 2 to 7 h after reversal of the nitrous oxide block were very effective in inactivating the mitotic factors. Cells collected at later times had decreased activity (Fig. 1). Furthermore, these inactivating factors were detectable as early as 1.5 h after reversal of the N_2O block when 10–15% of the cells were yet to complete mitosis. These data suggest that there are certain factors in G_1 cells that inhibit the MPA of the mitotic cell

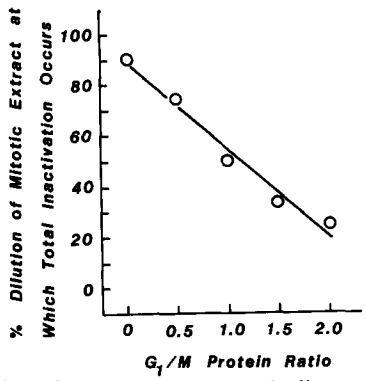

FIGURE 2 Inactivation of mitotic factors as a function of protein concentration in the G_1 cell extract. Early G_1 phase HeLa cells were obtained at 3 h after reversal of the N_2O block. G_1 cell extracts with various protein concentrations (~4–16 mg/ml) were prepared by varying the cell number while keeping the volume of the extraction buffer constant. Protein content of the mitotic cell extracts was kept constant by taking 4 × 10^7 cells/ml (~8 mg/ml) throughout these studies. Aliquots of G_1 cell extracts with various protein concentrations in relation to that of mitotic extract, i.e., 0.5, 1.0, 1.5, and 2.0, were separately mixed with mitotic extracts in various proportions and tested for MPA as described in the legend for Fig. 1.

extracts and we have termed them inhibitor(s) of mitotic factors (IMF).

Inactivation of Mitotic Factors by IMF is Dose Dependent

By increasing the protein content in G_1 extracts, inactivation of the mitotic extract could be obtained at lower dilutions (Fig. 2). The higher the G_1 protein content the lower the amount of G_1 extract needed to completely inactivate the mitotic factors, provided the concentration of mitotic proteins was kept constant.

Presence of IMF during S Phase of HeLa Cells

To determine whether the inhibitors of mitotic factors are present during other phases of the cell cycle, similar studies were carried out with extracts of HeLa cells synchronized in different stages of S and G_2. When extracts from early-, mid-, and late-S phase cells were mixed with mitotic extracts and then injected into oocytes to test for MPA, we observed that each of these S phase extracts did not neutralize the mitotic factors but delayed GVBD by 4.5 h. The method of synchronization into S phase (by reversal of second thymidine block or reversal of N_2O block) or of harvesting cells (by trypsinization or scraping) did not make any difference. However, increasing the protein concentration of the S phase extracts threefold (by taking more cells in the same volume of extraction buffer) increased the inhibitory activity of these extracts. Extracts from early S phase cells were more effective in neutralizing the activity of the mitotic factors than those from either mid- or late-S phase cells (Fig. 3). No significant IMF activity was observed in late-S phase cell extracts even when S phase/mitotic cell protein ratio was increased to 5:1. Extracts from early-, mid-, or late-G_2 phase HeLa cells also had no inhibitory effect on the mitotic factors.

Activation of IMF in Quiescent (G_0) WI-38 Human Fibroblasts

Extracts of WI-38 cells collected 7 d after they had reached confluency were mixed with extracts of mitotic HeLa cells in various proportions as explained above and the mixtures were injected into oocytes to test for MPA. Extracts from quiescent

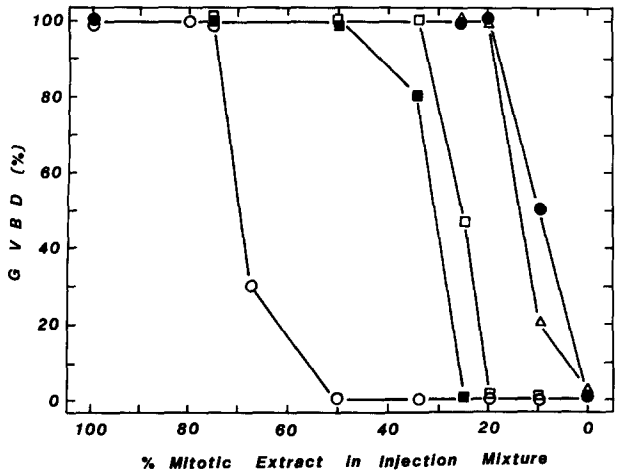

FIGURE 3 Effect of S phase cell extracts on the MPA of mitotic extracts. HeLa cells were synchronized in S phase as described under Materials and Methods. Early-, mid-, and late-S phase cells were collected at 2, 4, and 6 h, respectively, after reversal of the second thymidine block. Extracts from these S phase cells were prepared using 12–15 × 10^7 cells/ml, with a protein concentration of 22–27 mg/ml, whereas the mitotic extract was prepared by using 4 × 10^7 mitotic cells/ml, with ~8 mg/ml of protein, thus giving an S-phase/mitotic protein ratio of 3:1. Aliquots from each of these S phase extracts were separately mixed with mitotic extracts in various proportions and mixtures were injected into oocytes to test for MPA as explained in the legend to Fig. 1. ●, dilution with buffer (negative control); O, dilution with mid- G_1 cell extract as in Fig. 1 (positive control); dilution with extracts from early, ■, mid-, □, and late, △, S phase cells.

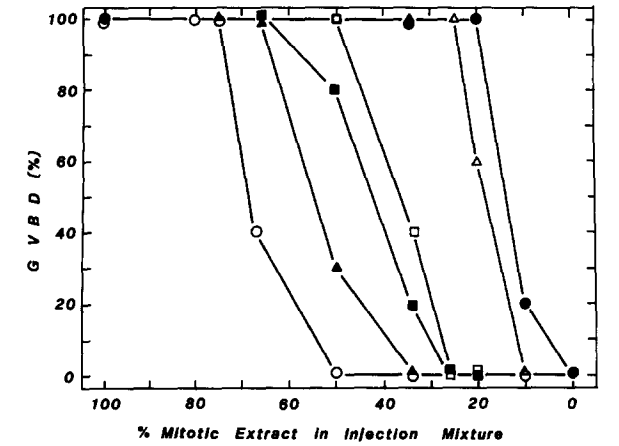

FIGURE 4 Activation of IMF in quiescent (G_0) WI-38 human diploid fibroblasts by UV irradiation. WI-38 cells in G_0 phase were collected at 7–10 d after they had reached confluency. G_0 cells were UV-irradiated for 10 s at 90 ergs cm^{-2} sec^{-1} and incubated for various times in the presence or absence of cycloheximide (25 μg/ml), arabinosylcytosine (10^{-4} M), or hydroxyurea (10^{-2} M). Extracts from the control and treated G_0 cells were prepared so as to contain a protein concentration equal to that of mitotic extracts (~8 mg/ml). Extracts of G_0 cells from the different treatments were separately mixed with mitotic extracts and tested for MPA as in Fig. 1 legend. ●, dilution with buffer (negative control); O, dilution with mid G_1 cell extract (positive control); △, extracts from untreated G_0 cells pretreated with Ara-C and hydroxyurea; extracts from G_0 cells, UV-irradiated and incubated in the presence or absence of cycloheximide for 2 h (□); and 4 h (■); extracts from G_0 cells, UV-irradiated and incubated for 2 h in the presence of Ara-C and hydroxyurea (▲). The data presented here are an average of two experiments.

cells exhibited minimal inhibitory effects on mitotic factors. However, UV irradiation (which is known to induce chromosome decondensation and unscheduled DNA synthesis [7, 12, 36]) of G_0 cells, and subsequent incubation for 2–4 h increased the inhibitory activity of extracts from these cells (Fig. 4). The presence of cycloheximide during incubation had no effect on IMF activity. IMF activity was further enhanced if hydroxyurea and arabinosylcytosine were added during incubation after UV irradiation.

Activation of IMF at Mitosis-G_1 Transition

To determine whether the IMF were newly synthesized or were pre-existing factors activated at the M-G_1 transition, we synchronized HeLa cells in mitosis by the N_2O block method and allowed them to divide in the presence or absence of cycloheximide. At 3 h after reversal of the mitotic block, when >95% of the cells were in G_1, extracts were made from the control and cycloheximide-treated cultures, separately mixed with mitotic extracts in various proportions, and tested for MPA. The extracts from cells that were allowed to divide in the presence of cycloheximide were as inhibitory as those from the control G_1 cells (data not shown). These experiments indicate that the IMF are not newly synthesized but rather are activated at the time of cell division.

Preliminary Characterization of the IMF

Our earlier studies (1, 2, 40) have shown that the mitotic factors of HeLa cells are highly sensitive to Ca^{2+}. These factors are completely inactivated in the presence of 1 mM Ca^{2+}. It is, therefore, possible that the inactivation of mitotic factors at the end of mitosis could be due to an excess release of calcium into the cytoplasm. To rule out this possibility two sets of experiments were performed. In the first set, a freshly prepared G_1 cell extract was titrated with increasing concen-

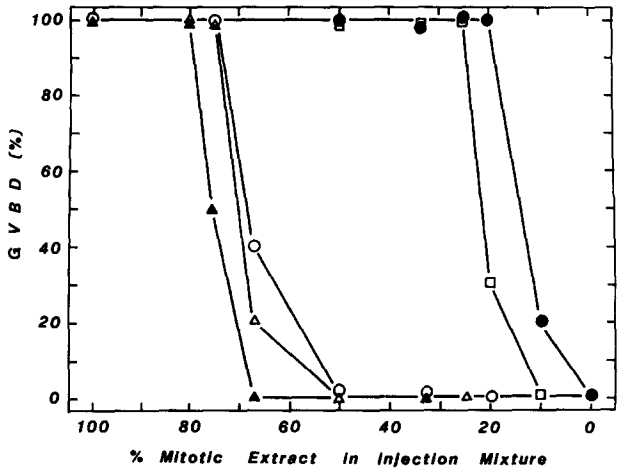

FIGURE 5 Effect of chelating agents on the IMF activity. Extracts from early-G_1 HeLa cells were prepared as described in legends to Figs. 1 and 2 and incubated for 1 h at 4°C with various concentrations of EGTA or EDTA (1 mM–5 mM). These EGTA- or EDTA-treated G_1 cell extracts were separately mixed with extracts from mitotic HeLa cells and tested for MPA as described in the legend to Fig. 1. ●, buffer (negative control); O, HeLa G_1 cell extracts (positive control); □, buffer containing 5 mM EGTA or EDTA; △, HeLa G_1 cell extracts incubated with 1, 2, 3, or 4 mM EGTA or EDTA prior to being mixed with mitotic extracts; ▲, HeLa G_1 cell extracts incubated with 5 mM EGTA or EDTA. These data represent an average of two experiments.

TABLE I

Preliminary Characterization of IMF

Treatment of G_1 cell extract	Relative IMF activity
	%*
None	100
Papain‡	0
RNase§	100
Temperature‖	
0°C for 2 d	80
−70°C for 2 mo	100
25–65°C for 15 min	100
75°C for 15 min	75
100°C for 15 min	0
pH¶	
4.0	20
5.0–5.5	75
6.0–8.0	100
8.5–10.0	75–80

* A freshly prepared mid-G_1 cell extract from 8×10^7 cells/ml was mixed with mitotic extract from 4×10^7 cells/ml to give a mitotic/G_1 protein ratio of 1:1, and the mixture was injected into *Xenopus laevis* oocytes for MPA determination. A mixture of mitotic extract with extraction buffer served as control. If the G_1 cell extract completely inactivated the MPA of the mitotic extract at a 50% dilution, i.e., a protein ratio of 1:1, then the activity of the G_1 cell extract was considered to be 100%. An average of 10 oocytes was injected for each sample.

‡ G_1 cell extract was treated with the protease papain (500 μg/ml) in extraction buffer containing cystein (5 mM) and β-mercaptoethanol (0.05 mM) for 1 h at 25°C. Antipain (80 μg/ml) was then added for 15 min at 25°C to neutralize the excess papain before being mixed with mitotic extract in various proportions and eventually injected into oocytes for MPA determination. In these experiments we made certain that papain activity was completely neutralized by antipain so that it could not inactivate the mitotic factors, which are known to be proteins.

§ G_1 cell extracts were incubated with RNase (1.5 U/ml) at 25°C for 1 h. At the end of incubation, G_1 extracts were mixed with mitotic extracts and tested for MPA as above.

‖ G_1 cell extracts were incubated at different temperatures for 15 min and at the end of the incubation extracts were centrifuged at 10,000 *g* for 15 min at 4°C to remove any precipitate before being mixed with mitotic extract for injection into oocytes.

¶ G_1 cell extracts were dialyzed overnight against buffers of different pH with three changes each and redialyzed for 12 h against the extraction buffer (pH 6.5) before the mixing experiments were carried out to test the activity, as explained above.

trations of the Ca^{2+}-chelating agent EGTA before the mixing experiments with mitotic extracts were performed. In control experiments, the mitotic cell extract was treated with identical concentrations of EGTA and tested for MPA. The difference between the inhibitory activities of the control and the EGTA-treated G_1 cell extracts was insignificant (Fig. 5). Similar results were obtained when EDTA instead of EGTA was used. In the second set of experiments, G_1 cell extract was dialyzed through a bag with a cut-off limit of either 3,500 or 12,000 mol wt with a buffer lacking Ca^{2+}, EDTA, or EGTA. Dialysis had no effect on the inhibitory activity of the G_1 cell extract when it was later mixed with mitotic cell extract (data not shown).

Effect of Proteases and RNase

To test whether the activity of IMF was protease sensitive, the G_1 cell extract was incubated with a protease, Papain or trypsin, for 1 h at 25°C. Antipain or trypsin inhibitor was then added to neutralize the excess protease before mixing it with the mitotic cell extracts. As shown in Table I, treatment with papain resulted in complete inactivation of the inhibitors of mitotic factors. Control experiments performed showed that the amount of antipain used was enough to inhibit all papain activity. Injection of extraction buffer containing papain or antipain did not show any MPA.

Treatment of G_1 cell extract with RNase (1.5 U/ml) at 25°C for 1 h before performing the mixing experiments did not result in any inactivation of these factors (Table I). We have already shown that RNase has no effect on the MPA of mitotic factors (40). RNase at the concentration tested was not toxic to oocytes and did not induce any GVBD when injected alone.

Effect of Temperature

Incubation of the G_1 extract at temperatures up to 65°C for 15 min had no effect on the IMF activity (Table I). At 75°C for 15 min, a slight precipitation and a 25% loss in the activity of these factors was observed. At 100°C for 15 min, these factors were completely inactivated.

Effect of pH

The IMF were found to be active over a broad range of pH, and the pH optimum was found to be between 7 and 8. Very little activity was seen below pH 5 (Table I). These properties clearly distinguish the IMF from mitotic factors, which seem to be more stable at low pH.

DISCUSSION

In this study, we have attempted to determine the fate of the chromosome-bound mitotic factors at the end of mitosis. We have demonstrated the existence of certain factors in G_1 cells that can inactivate or inhibit the mitotic factors and that have tentatively been named inhibitors of mitotic factors (IMF). The bioassay system for IMF involves the mixing of extracts from interphase (G_1, S, or G_2) HeLa cells with mitotic cell extracts in various proportions and injecting the mixture into immature *Xenopus* oocytes to determine the extent of inactivation of the mitotic factors as indicated by a decrease in the frequency of GVBD among the injected oocytes. Mitotic extracts diluted with the extraction buffer (with or without BSA) in corresponding proportions served as controls. This method of measuring IMF activity, though indirect, should be useful for the isolation and characterization of these factors.

Using this assay, we have shown that the levels of IMF fluctuate during the cell cycle in a cyclical pattern. These factors are activated at telophase, when the process of chromosome decondensation begins, and are present at relatively high levels throughout the G_1 period. There appears to be an inverse linear relationship between the protein content of the G_1 cell extracts and the dilution ratio of mitotic/G_1 phase extracts at which mitotic factors are inactivated (Fig. 2). These factors reach their minimum level during S phase and are absent during G_2 phase, when mitotic factors are known to accumulate (1, 39).

The preliminary characterization of the IMF reported here suggests that these factors are nondialyzable, nonhistone proteins sensitive to inactivation by proteases, but not by RNase, EGTA, or EDTA. It is unlikely that the IMF activity could be due to a protease as three different protease inhibitors (phenylmethylsulfonyl fluoride, antipain, and trypsin inhibitor) used in our study had no effect on IMF activity. Unlike the mitotic factors, IMF are heat stable (15 min at 65°C). They are stable over a broad pH range (6.0–10.0) but are extremely sensitive to low pH (below 5.0), and the apparent

molecular weight is >12,000. Some of these characteristics distinguish them from the mitotic factors (40). The pH dependency of these factors is also in agreement with the results of the early cell fusion experiments of Obara and co-workers (21–23), which suggested that high pH favored "telophasing" and low pH "prophasing" or premature chromosome condensation. Our own studies (Hittelman and Rao, unpublished data) show that the frequency of premature chromosome condensation induction is much higher if a low pH is maintained during the collection of mitotic cells and subsequent fusion procedures. Thus, it appears that at low pH, the mitotic factors are active, whereas the G_1 factors are either inactive or less active.

The existence of a chromosome condensation cycle within the life cycle of mammalian cells was first proposed by Mazia (16) and has subsequently been substantiated by various investigators using different experimental approaches (8–11, 17, 18, 20, 24, 25, 30, 33, 35–37, 41, 42). According to these studies the decondensation of chromatin takes place throughout G_1 and until the beginning of S phase, at which time DNA replication is initiated. Following replication, the chromatin recondenses during late S and G_2 periods and reaches a maximum level of condensation at mitosis in the form of chromosomes. Regulation of this sequence of events by cytoplasmic factors has been suggested by the experiments of Rao and Johnson (32) and Johnson and Rao (13). Using the technique of premature chromosome condensation, they have shown that chromosome condensation factors are present in mitotic cells (14, 31).

Subsequently, we have shown that microinjection of mitotic extracts into *Xenopus* oocytes causes meiotic maturation, i.e., GVBD and chromosome condensation, a process similar to premature chromosome condensation (39). Using this system we have demonstrated that the mitotic factors are nondialyzable, heat- and Ca^{2+}-sensitive, magnesium-dependent, nonhistone chromatin-binding proteins with an approximate molecular weight of 100,000 (1, 2, 40).

The accumulation of mitotic factors during G_2 and mitosis (1, 2, 39, 40) is closely correlated with a progressive increase in the chromosome condensation. During metaphase, when the chromosomes are most condensed, the levels of mitotic factors are the highest. What happens to the mitotic factors at the end of mitosis? The results of this study suggest that certain factors (proteins) are activated at the mitosis-G_1 transition that inactivate the mitotic factors in situ. The IMF are present throughout G_1 and the early part of S phase in HeLa cells. Furthermore, our recent in vitro studies with IMF and mitotic factors suggest that the IMF specifically inactivate the mitotic factors probably by binding to their active site and forming an inert complex (3).

As mentioned earlier, the fusion of a mitotic cell with an interphase cell results in a rapid chromosome condensation and the dissolution of the nuclear envelope of the interphase cell within 45 min after fusion. This phenomenon has been termed premature chromosome condensation (14) or "prophasing" (15). The ability of a mitotic cell to induce premature chromosome condensation in an interphase nucleus depends largely on the ratio of mitotic to interphase nuclei in the cell at the time of fusion. Very low frequency or no induction of premature chromosome condensation was observed in tri-, or tetranucleates containing one mitotic and two or three G_1 nuclei (14). Furthermore. Obara and co-workers (21–23) have reported that in certain cases in these multinucleate cells containing higher ratio of interphase cells, a membrane is formed around the metaphase chromosomes. Because of its resemblance to the process occurring in normal telophase, this process has been termed "telophasing" (21–23). They have also demonstrated that the larger the ratio of interphase nuclei to chromosomes in the fused cells the greater the possibility that "telophasing" would occur rather than prophasing. Moreover, we have observed that if the mitotic cells are held for a prolonged period in Colcemid, their ability to induce premature chromosome condensation in interphase cells is greatly diminished (unpublished observations). The present study seems to provide an explanation for the failure of mitotic cells to induce premature chromosome condensation in multinucleate cells when the interphase to mitotic ratio is >2.

Our data reported here indicate that the IMF are activated at telophase and are present throughout G_1 period thus coinciding with the process of chromosome decondensation which is known to begin at telophase and continue throughout G_1 phase. It is tempting to speculate that the IMF which are antagonistic to mitotic factors may serve the reverse function of the mitotic factors, i.e., if the mitotic factors are involved in chromosome condensation, the IMF may be involved in chromosome decondensation. This proposition is further strengthened by the following observations: (*a*) Noncycling G_0 phase human fibroblasts, in which the chromatin is more condensed than in cycling G_1 cells (9), contain little or no IMF (Fig. 4). However, IMF can be activated in these cells by UV irradiation which causes chromosome decondensation and DNA repair synthesis (7, 12, 36). (*b*) We have recently observed that the UV-induced chromosome decondensation in mitotic HeLa cells is associated with inactivation of mitotic factors and the induction of IMF (3). (*c*) In cell fusion experiments, Rao and Johnson (32) and Rao et al. (33) observed that the entry of G_2 cells into mitosis is delayed after fusion with G_1 or S phase cells until G_1 or S phase nuclei in the heterophasic binucleate cells have completed DNA synthesis and subsequently both nuclei have entered mitosis synchronously. They speculated that the G_1 and S phase components were causing decondensation of chromatin in G_2 nuclei, thus blocking them from entering mitosis. The present study seems to provide experimental evidence to support that assumption. Taken together, all these observations suggest that IMF may play a role in the regulation of chromosome decondensation. However, a direct relationship between IMF and chromosome decondensation remains to be established.

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