Chromosome-bound mitotic factors: release by endonucleases

Ramesh C. Adlakha¹, Chintaman G. Sahasrabudhe², David A. Wright³, William F. Lindsey³, Marion L. Smith¹ and Potu N. Rao¹

Departments of ¹Developmental Therapeutics, ²Pathology, and ³Genetics, The University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston, Houston, TX 77030, USA

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ABSTRACT

Additional evidence is presented to support our recently reported conclusion that the mitotic factors of mammalian cells, which induce germinal vesicle breakdown and chromosome condensation when injected into fully grown *Xenopus laevis* oocytes, are localized on metaphase chromosomes. Chromosomes isolated from mitotic HeLa cells were further purified on sucrose gradients and digested for varying periods with either the micrococcal nuclease or DNase II. At each time point of digestion the amount of mitotic factors released was determined by injecting a supernatant of these fractions, obtained by high-speed centrifugation, into oocytes. The amount of DNA rendered acid soluble under the conditions of digestion used was 3% to 5% of the total chromosomal DNA. The extent of release of mitotic factors with both nucleases was estimated to be about 30% to 40% as evidenced by the reextraction of the undigested chromosomal pellet with 0.2 M NaCl. Similar results were obtained when nuclei from G2 cells were digested under identical conditions. The release of these chromosome-bound mitotic factors by mild digestion with these nucleases though only partial, clearly demonstrates that a significant proportion of these factors are localized on metaphase chromosomes.

INTRODUCTION

The presence of specific factors for the transformation of interphase nuclei into discrete chromosomes in mitotic cells and their ability to induce this event in interphase cells prematurely has been demonstrated by cell fusion experiments in various systems (1, 2). However, the nature of these factors and the mechanism of their action remains to be elucidated. It has recently been shown that when mitotic extracts from mammalian cells (3-7) were injected into *Xenopus laevis* oocytes, they exhibited maturation-promoting activity (MPA) as evidenced by germinal vesicle breakdown (GVBD) and condensation of chromosomes. Meiotic maturation in the fully grown amphibian oocytes can be induced by hormones like progesterone and insulin (8-10), surface-acting agents like lanthanum (11), local anaesthetics, cationic drugs (12), and sulfhydryl reagents (13), and more recently by the injection of calcium-calmodulin complex (14). During the process of maturation, a maturation promoting factor (MPF) is synthe-

sized in the oocyte cytoplasm; it is capable of inducing maturation when injected into fully grown oocytes (15).

In our attempt to characterize the mitotic factors by using amphibian oocytes as a bioassay system, we recently demonstrated (16) that these factors preferentially bind to chromatin as soon as they are synthesized during G2 phase, and as the cell synthesizes more of these factors in preparation for mitosis, increasing amounts of them are retained in the cytoplasm. If the mitotic factors are bound to chromosomes, can they be released by digestion with endonucleases? The results of this study indicate that a partial release, (about 30-40%) of these factors is achieved by a mild digestion of the isolated metaphase chromosomes with either micrococcal nuclease or DNase II. The mitotic factors not released by nuclease digestion can be extracted by 0.2 M NaCl buffer. An abstract of this study has appeared elsewhere (17).

MATERIALS AND METHODS

Methyl [^3H] thymidine (sp. activity 6.7 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Micrococcal nuclease and DNase II were obtained from Worthington Biochemicals (Freehold, New Jersey). Most other chemical products were mainly purchased from Sigma Chemical Company (St. Louis, MO).

Cells and Cell Synchrony

HeLa cells were grown as suspension cultures in spinner flasks at 37°C in Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum, sodium pyruvate, nonessential amino acids, glutamine, and penicillin-streptomycin mixture in a humidified 5% CO₂ atmosphere (18).

To obtain mitotic cells, a random population of HeLa cells from spinner cultures was plated in 1585 cm² borosilicate roller bottles (Bellco Glass Co., Vineland, NJ) at a density of 120 to 160 x 10⁶ cells. The bottles were gassed for 1 min with sterile 5% CO₂/95% air at 10 psi (~70 kPa), closed tightly, and kept at 37°C in a roller apparatus at 0.2 rpm. Media was changed the next morning and bottles gassed again. The cells were partially synchronized in S phase by a single thymidine block (2.5 mM) for about 20 h. Mitotic cells were obtained by reversing the thymidine block by washing and incubating cells in fresh medium containing Colcemid (0.05 μ g/ml) for another 20 h at 37°C. The rounded and the loosely attached mitotic cells were selectively detached by gently shaking and twirling the roller bottle. This procedure routinely yielded a population with a mitotic index of 90-95%. Cells from four roller bottles were usually plated at one time, and the yield

from these four varied between 1.0 and 1.2×10^9 cells.

Mitotic cells labeled with ^3H -dThd were obtained by adding $0.05 \mu\text{Ci/ml}$ of [^3H] thymidine to the roller bottles during the cell synchronization procedures. HeLa cells synchronized in S and G2 phases were obtained by harvesting cells at 1 h and 9 h, respectively, after the reversal of the second excess thymidine block as described previously (16, 19).

Preparation of Cell Extracts

The mitotic, S phase, and G2 phase cells were collected in cold by centrifugation at 1000 rpm for 5 min. After 3 washings with MEM without serum at 4°C , the cells were resuspended in the buffer developed by Blumenthal *et al* (20) [15 mM Tris-HCl containing 60 mM KCl , 15 mM NaCl , $0.5 \text{ mM spermidine}$, 0.15 mM spermine , $15 \text{ mM } \beta\text{-mercaptoethanol}$, 2 mM EDTA , $0.5 \text{ mM ethyleneglycol bis (} \beta\text{aminoethyl ether)-N,N,N',N'tetra acetic acid (EGTA)]$], supplemented with a protease inhibitor ($1 \text{ mM phenylmethyl sulfonylfluoride}$, PMSF) and phosphatase inhibitors, viz., 1 mM ATP , 5 mM NaF , $5 \text{ mM sodium } \beta\text{-glycerolphosphate}$, and 0.25 M sucrose at pH 7.4. Earlier studies have shown that these inhibitors have a stabilizing effect on the mitotic factors (16). The cells were lysed by several passages through a 23-gauge syringe as described previously (16), and the chromosomal pellet was washed once again with the same buffer and then resuspended in the digestion buffer [0.35 M sucrose , 10 mM Tris-HCl (pH 7.4) 25 mM KCl , 5 mM MgCl_2 , 0.2 mM CaCl_2 , 1 mM ATP , 1 mM PMSF , 5 mM NaF , and $5 \text{ mM sodium } \beta\text{-glycerolphosphate}$] and layered on 20% sucrose. After centrifugation in a SW27 swinging bucket rotor at $10,000 \times g$ for 30 min, the chromosomes were collected.

Chromosome Fractionation and Digestion with Nucleases

Chromosomes were fractionated in a way similar to that of Bloom and Anderson (21) for chromatin. Chromosomes or nuclei purified on sucrose gradients as mentioned above were incubated at 37°C in the digestion buffer containing 200 units/ml of micrococcal nuclease or 100 units/ml of DNase II for varying periods of time. The digestion buffer for micrococcal nuclease contained 0.2 mM CaCl_2 , and digestion was carried out according to the procedure described (21, 22). The digestion with DNase II was done in the presence of MgCl_2 as described by other groups (23, 24). Following digestion, the various fractions were chilled on ice and the reaction terminated by the addition of 100 mM EGTA to micrococcal nuclease-digested fractions and 100 mM EDTA to DNase II-digested fractions to a final concentration of 2 mM . Each fraction was centrifuged at $10,000 \times g$ for 10 min at 4°C . Supernatants were collected and assayed directly for maturation promoting activity. The undi-

gested pellet obtained after centrifugation of the various fractions for 5 min at 10,000 x g was washed once with the same buffer and then extracted with high-salt buffer as described (16).

Assay for Digestion

At each of the indicated times of digestion, an aliquot of the nuclear or chromosomal suspension was withdrawn and the disintegrations counted in a Packard liquid scintillation counter to determine the total amount of radioactivity in the nuclear or chromosomal DNA. Also at each digestion time, 7.0 N perchloric acid was added to an aliquot until the acid concentration reached 0.8 N, and the aliquot was kept at 4°C for 15 min and centrifuged; the amount of DNA rendered acid soluble was obtained by counting disintegrations in the supernatant.

Preparation of *Xenopus* oocytes and Assay for Maturation-Promoting Activity

All the procedures employed for the preparation of the oocytes and assay of the cell extracts for MPA by microinjection into oocytes were essentially the same as described previously (16). The protein content of the extracts was assayed with Bio-Rad dye reagent, using crystalline bovine serum albumin as the standard (25).

RESULTS

Release of Mitotic Factors by Digestion of Metaphase Chromosomes with Micrococcal Nuclease and DNase II

The relative amounts of chromosomal DNA rendered acid soluble in various fractions during a typical time course of digestion are shown in Figure 1. The rate of DNA rendered acid soluble was biphasic, with a very rapid increase during the first 3 min of digestion followed by a slow, gradual increase for at least 30 min. The kinetics of release were identical for both nucleases.

The data from several digestion experiments on the kinetics of the release of mitotic factors (% GVBD) of the various chromosome-digested fractions with the two nucleases are presented in Figure 2. A very good correlation between the pattern of release of mitotic factors and the amount of DNA rendered acid soluble was observed, particularly in the range of 1.25-2.0% in case of DNase II and 2.25-3.0% in case of micrococcal nuclease (Fig. 3). Mitotic factors were released rapidly until approximately 3% of the DNA was rendered acid soluble in the case of micrococcal nuclease and 2% in the case of DNase II. Much less mitotic factors were released thereafter. In fact, no appreciable amount of MPA was observed in fractions digested for 10 min or more. The loss of activity in these fractions is probably due to the longer incubation of the mitotic

Figure 1. Amount of DNA rendered acid soluble as a function of time of digestion of metaphase chromosomes with nucleases: Purified chromosomes from mitotic HeLa cells (40×10^6 cells/ml) labeled with [^3H] thymidine were digested with either 200 units/ml of micrococcal nuclease or 100 units/ml of DNase II. At each of the indicated times an aliquot of the chromosomal suspension was withdrawn and fractionated as described in Materials and Methods. An aliquot of each fraction was brought to a final concentration of 0.8 N perchloric acid, placed at 4°C for 15 min, and centrifuged, and DNA content was determined by measuring the radioactivity. The percentage of acid soluble DNA of the total chromosomal DNA at each time point of digestion is shown in the figure. $\circ$, micrococcal nuclease digestion; $\bullet$, DNase II digestion, and $\square$, control.

factors in a diluted form (app. 1:2 dilution) at 37°C in the presence of the enzymes. Our earlier results showed that incubation of even the crude mitotic extract (dilutable 1:3) for 10 min at 40°C or more results in practically complete inactivation of the mitotic factors (4).

Higher concentration of the enzymes, i.e., 400 units/ml of micrococcal nuclease or 200 units/ml of DNase II, did not significantly increase the amount of mitotic factors released or the amount of DNA rendered acid soluble.

High salt extraction of the chromosomal pellet releases all the MPA (16). A volume of 65 nl of this extract used for microinjection contained approximately 180–200 ng of protein. However supernatants after digestion of the same amount of chromosomes (i.e., from 40×10^6 mitotic HeLa cells) with the nuclease contained approximately 70–80 ng of protein which is nearly a third of the total MPA associated with the chromosomes (Table 1). Further extraction of the nuclease-digested chromosomes released the remaining mitotic proteins. Although these three extracts of chromosomes, i.e., high salt, nuclease digests and high salt extracts or nuclease-digested chromosomes, contained variable amounts of protein, but had similar MPA as indicated by serial dilutions (data not shown). These data suggest that about 30–40% of the total chromosome-bound mitotic factors were released

Figure 2. Kinetics of the release of mitotic factors (measured as % GVBD) as a function of time of digestion with nucleases: Chromosomes were digested with either micrococcal nuclease 200 units/ml or DNase II (100 units/ml) as explained in legend to Figure 1. At each of the indicated times of digestion an aliquot of the chromosomal suspension was withdrawn and after centrifugation at $100,000 \times g$ for 1 hr, 65 nl of the supernatant was injected into each oocyte and percent GVBD scored after 2-3 hrs. The undigested pellet at each time point of digestion was reextracted with 0.2 M NaCl buffer (for details see Materials and Methods) and an aliquot injected into oocytes to score the percent GVBD. ○, micrococcal nuclease digestion; ●, digestion with DNase II; □, control and ■, reextraction with high salt. An average of 25 oocytes were injected with each extract.

Figure 3. Correlation between the release of mitotic factors (measured as percent GVBD) and the amount of DNA rendered acid soluble during the first 5 min of digestion of metaphase chromosomes with nucleases. For details of digestion conditions and scoring of percent GVBD refer to legends to Figures 1 and 2, and Materials and Methods. ○, micrococcal nuclease digestion; and ●, digestion with DNase II.

by nuclease digestion. No mitotic factors were released when chromosomes were incubated with digestion buffer without the enzymes.

Release of Mitotic Factors from G2 Cells

Since our recent studies (16) have indicated the presence of MPA in the nuclear extracts of early-, mid-, and late G2 cells, we decided to determine whether this activity can be released by the digestion of the isolated G2 nuclei with micrococcal nuclease and DNase II. Here again, 30-40% of the chromatin-bound factors were released. The extent of release at the optimum digestion times are presented in Table I. The kinetics of release were very similar to those of the metaphase chromosomes.

The above experiments clearly indicate that the mitotic factors bound to chromosomes can be released, though only partially, by mild digestion with endonucleases. However, in order to rule out the possibility that the MPA exhibited by various digested fractions of chromosomes or nuclei was due to a slower sedimentation of the chromatin fragments, purified nuclei from S phase cells were digested under exactly identical conditions. As shown in Table 1, none of the fractions exhibited any measurable MPA.

DISCUSSION

In a recent study, we observed that a major portion of the mitotic factors (which induce GVBD and chromosome condensation when injected into fully grown oocytes) were localized on metaphase chromosomes (16). These factors become preferentially associated with chromatin as soon as they are synthesized. Our studies also indicated that these proteins associate with chromatin in relatively weak ionic bonds, since they are completely extractable in 0.2 M NaCl (3,4,6,7,16). These bonds are weaker than the ionic interactions between DNA and histones or nucleosomes and high mobility group (HMG) proteins. The fact that the nucleosomal structure remains intact under the salt conditions used for the extraction of these mitotic factors suggests that these proteins are probably extranucleosomal (26). The information about their spatial distribution, therefore, is important for revealing the metaphase chromosome structure.

The results presented here allow us to develop some ideas about the structural aspects of metaphase chromosomes. Analysis of the organization of DNA within the mitotic chromosomes has relied, in the past, upon electron microscopic observations. These studies have revealed a 10-nm unit fiber, which by specific folding is thought to generate the native fiber of 20-30 nm thickness (27, 28). It has been shown that although significant morphological changes occur within the chromatin fiber during mitosis, the basic subunit

Table 1

Release of mitotic factors by digestion of metaphase chromosomes or interphase nuclei with micrococcal nuclease (200 units/ml) and DNase II (100 units/ml)

Oocytes injected with supernatants after	Micrococcal Nuclease Digestion		DNase II Digestion	
	Protein Injected (ng)	GVBD Induction (%)	Proteins Injected (ng)	GVBD Induction (%)
Digestion of metaphase chromosomes	73	80	85	90
Nuclease-digested chromosomes extracted with high salt buffer	111	100	107	100
Digestion of nuclei of G ₂ cells	91	50	95	60
Nuclease-digested nuclei from G ₂ cells extracted with high salt buffer	97	73	90	80
Digestion of nuclei from 'S' phase cells	107	0	101	0
Nuclease-digested nuclei from S phase cells extracted with high salt buffer	123	0	115	0
Chromosomes extracted with digestion buffer without the enzyme	21	0	21	0
Digestion buffer alone	0	0	0	0

(a) Data from several digestion experiments as described under Materials and Methods and legends to fig. 1 and 2.

(b) A cell density of 40×10^6 to 60×10^6 cells/ml was used for preparation of these extracts and 65 nl of the extracts was injected into each oocyte.

(c) An average of 25 oocytes were injected with each extract.

(d) The data in this table refer to the maximum release of mitotic factors at the optimum digestion time of 3.5 min in case of micrococcal nuclease and 2 min for the DNase II.

structure of the chromatin fiber is retained in the mitotic chromosomes (28).

Based on our data, we propose that the mitotic factors bind to the native chromatin fiber. Binding of these factors induces tight packaging of chromatin fiber into metaphase chromosomes. The compaction of the chromatin fibers by the mitotic factors could be achieved in one of two ways: (1) The mitotic factors newly synthesized during G₂ (4, 16) bind to the chromatin directly and bring about its condensation. (2) The mitotic factors become associated with chromatin and modify (e.g., phosphorylation or acetylation) the structural proteins, which are a permanent constituent of chromatin, to induce chromosome condensation. Which of these two modes is operational in the cell remains to be elucidated.

However, in the case of Xenopus oocytes, as yet, there is no evidence for the direct association of mitotic factors with the chromosomes. It is likely that the mitotic factors when injected may be activating the oocyte maturation promoting factors that exist in an inactive form (16).

The relatively low level of DNA rendered acid soluble in the present study is probably due to (1) poor accessibility of inter- and intra-nucleosomal DNA to endonucleases due to compactness of chromatin fiber, (2) the use of suboptimal conditions of digestion, especially with regard to pH and Ca²⁺ concentrations, to prevent inactivation of mitotic factors, (3) and it is possible that some protease released during the preparation of chromosomes and the digestion process could inactivate the nucleases as well as the mitotic factors. However, this possibility is less likely because of the presence of PMSF in the isolation and digestion buffers. Furthermore, it has been observed that when low levels of chromosomal DNA (2-5%) are made acid soluble, only internucleosomal cleavages occur (29). Thus, under the mild conditions of digestion of metaphase chromosomes employed in this study, mostly large chromatin fragments containing polynucleosomal chains are released which pellet down on acid precipitation.

In the present study we observed that when metaphase chromosomes were digested with endonucleases, only 30%-40% of chromosome-bound mitotic factors (as revealed by MPA in Xenopus oocytes) were released. This low release could be due to nonrandom digestion of metaphase chromosomes under the mild conditions of digestion used. The observation of G banding patterns in acid-fixed chromosomes exposed to nucleases under similar conditions suggests the non-random nature of digestion (30). However, the mitotic factors still remaining bound to the partially digested chromosomes could be extracted with 0.2 M NaCl.

The release of the mitotic factors by endonucleases from the metaphase

chromosomes and G₂ nuclei further confirms our earlier observation that these factors are associated with chromatin.

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