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# Ionic Control of Chromosome Architecture in Living and Permeabilized Cells

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**Studies with isolated chromatin show that higher order chromosome architecture can be regulated by ionic conditions; however, the physiological relevance of these findings remains unknown. In the present study, chromosome architecture was analyzed *in situ* in living and detergent-extracted cells exposed to different ionic conditions. In intact mitotic endothelial cells, chromosomes instantly unfolded as detected by phase contrast microscopy when the salt concentration in the culture medium was increased from 110 to 410 mM NaCl or from 0 to 65 mM $MgCl_2$. When the ions were removed and the preexisting culture conditions were restored, chromosomes refolded into their original shapes and subsequently underwent mitotic division. Similar reversible effects were observed on nucleolar structure in living interphase cells as well as on mitotic chromosomes exposed to high salt after cell membranes were removed by treatment with Triton X-100. This permeabilized mitotic cell model was then used to identify proteins that remained tightly associated with chromatin during the ion-driven chromosome unfolding–refolding cycle and which therefore could be important for maintenance of chromosome structure. Under these conditions in which disassembled chromosomes retained their ability to fully recondense, more than 95% of Topoisomerase I was extracted whereas approximately 25% of Topoisomerase II$\alpha$ and 50% of Histone H1 remained tightly associated with chromatin. These data demonstrate the sensitivity of chromosome structure to variations in ionic concentration *in situ* and suggest that there are at least two distinct pools of Histone H1 and Topoisomerase II$\alpha$ associated with chromatin during mitosis, one of which may be required for chromosome compaction.**



***Key Words:*** **chromosome assembly; topoisomerase; histone; chromatin structure.**

## INTRODUCTION

Chromatin architecture is important for many aspects of cell regulation, including transcription, replication, recombination, and chromosome condensation and decondensation [1–4]. Yet, the mechanism by which higher order chromatin structure is established and remodeled remains poorly understood. Research on chromatin organization in mammalian cells is commonly carried out using material that is chemically treated and thus has lost the normal dynamic remodeling capability of living chromosomes. However, recent studies have revealed that when intact chromosomes are physically removed from living cells using microsurgery, they retain the ability to undergo reversible decondensation and recondensation in response to changes in salt conditions [5]. Still, the physiological relevance of this dynamic remodeling of chromatin remains unclear since these results were obtained with chromosomes that were physically removed from their normal intracellular context.

Topoisomerase I (Topo I), Topoisomerase II$\alpha$ (Topo II), and Histone H1 are all proteins which are associated with chromatin during mitosis and which have been suggested to provide structural functions [6–11]. These proteins were also shown to induce chromosomes that were decondensed by protease treatment to recondense *in vitro* [5, 12]. Topo II exerted this condensing effect independently of its DNA-unwinding activity. The finding that Topo II localizes at the interface between chromatin loops and the nuclear scaffold further supports the concept that this mechanoenzyme may play a nonenzymatic role in the establishment and maintenance of compacted chromosome form [13–16]. This view, however, remains controversial. For example, when Topo II protein was microinjected into *Drosophila* embryos, most of the enzyme was released from the nuclei after anaphase, suggesting that Topo II is not critical for maintenance of chromosome structure [17]. Also, Topo II can be extracted from *Xenopus* chromosomes under mild ionic conditions without altering chromosome morphology [18]. In the same *Xenopus* system, Histone H1 was shown not to be required for chromosome condensation [19].

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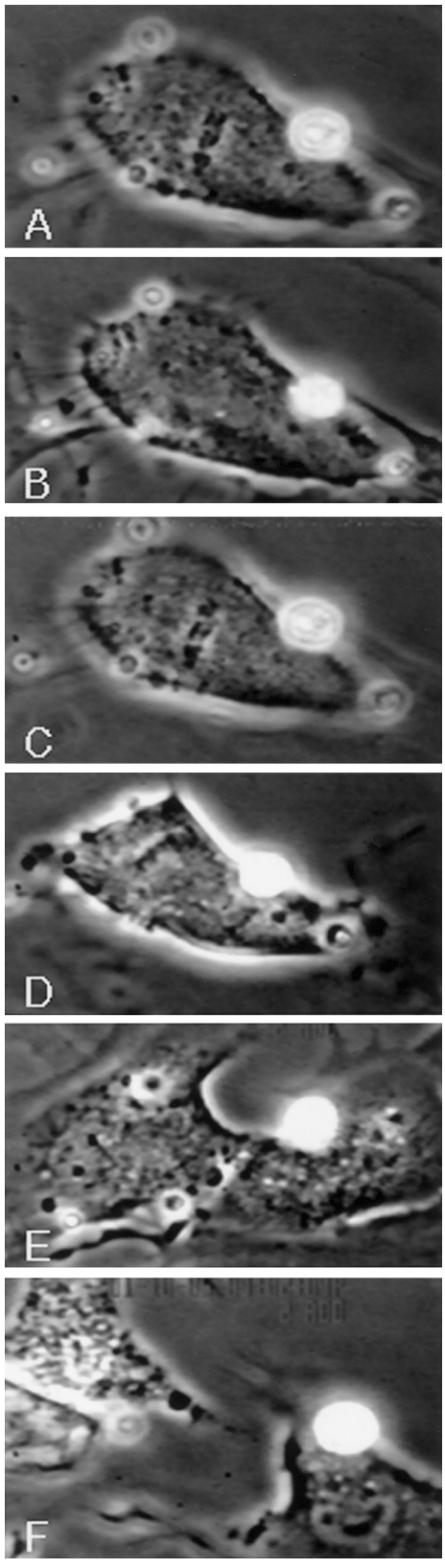


**FIG. 1.** Effect of varying ionic strength on a living mitotic cell. (A) Early anaphase cell before salt addition—chromosomes are aligned at the metaphase plate in the center of the cell. (B) The same cell—seconds after raising salt concentration in the medium to 65 mM $MgCl_2$—chromosomes unfold and become phase lucent. (C) The same cell 30 s after salts were washed away—chromosomes recover their preexisting shape, density, and position. (D–F) The salt-treated cell proceeds normally through mitosis and divides into two daughter cells (×1000).

In the present study, we examined the effects of varying ionic conditions on chromosome structure *in situ*, within nuclei inside living and detergent-permeabilized cells. We found that whole chromosomes can be induced to reversibly decondense and recondense by respectively increasing and decreasing salt concentrations in the culture medium under both conditions. We then used this model system to identify proteins that remain tightly associated with chromosomes after salt extraction and thus which may mediate the observed ion-induced chromosome remodeling. Using this approach, we now show that bovine endothelial cells contain two pools of Topo II and Histone H1. The reversible chromosome unfolding induced by high salt is accompanied by loss of approximately 75% of Topo II and 50% of Histone H1. However, salt-resistant Topo II and Histone H1 proteins remain tightly associated with the recondensed chromosomes. These results suggest that large-scale changes in chromatin structure may be driven by shifts in the balance of electrostatic charges within chromosomes and that subpopulations of chromosome-associated Topo II and histone H1 are well positioned to mediate these effects.

## MATERIALS AND METHODS

*Cell culture.* Bovine capillary endothelial cells were cultured on gelatin-coated glass coverslips in 30-mm tissue culture dishes (Falcon). Cell culture medium was DMEM (Gibco, BRL) supplemented with 10% calf serum (Hyclone), 25 mM Hepes (pH 7.4; JRH Biosciences) and 5 ng/ml basic fibroblast growth factor, as described [20]. The viability of salt-treated and untreated cells was measured by cell counting 24 h after ionic treatment, using a Mark Zf Coulter Counter. Alternatively, 9 h after salt treatment, cells were incubated with the thymidine analogue, the BrdU (5-bromo 2′-deoxyuridine, Amersham) for 5 h, fixed with ethanol/acetic acid/water solution (90:5:5), and incubated with murine anti-BrdU antibody (Amersham), followed by anti-mouse biotinylated goat secondary antibody and avidin-Texas Red (Vector). Nuclei were counterstained with DAPI, and BrdU-positive nuclei were counted through a Nikon Diaphot inverted microscope.

*Reversible ion-induced chromosome disassembly.* Chromosome disassembly was induced in intact living cells by raising the concentration of $MgCl_2$ from 0 to 65 mM, or of NaCl from 110 to 410 mM in DMEM medium, for 60–90 s. Permeabilized cells were prepared by 1 min treatment with 0.5% Triton X-100 in buffer A (20 mM Hepes, pH 7.4; 110 mM potassium acetate; 2 mM magnesium acetate; 0.5 mM EGTA) adapted from [21]. Cells were washed and incubated for 20 min in buffer A with 6 mM $MgCl_2$ in the presence or absence of 2.5 mM AMP-PNP (5′-adenylylimidodiphosphate; Sigma), a nonhydrolysable analogue of ATP. Chromosome unfolding in detergent-permeabilized cells was induced by adding an equal volume of buffer A with 130 mM $MgCl_2$, 710 mM NaCl, or 600 mM KCL to cells in 1 ml

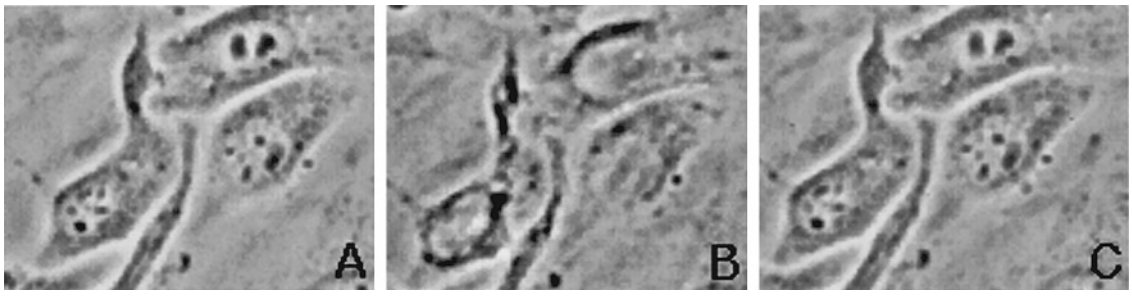


**FIG. 2.** Effect of varying ionic strength on living interphase endothelial cells. (A) Phase dense nucleoli are clearly visible within the nuclei of cells prior to salt treatment. (B) Same cells 60 s after addition of 65 mM $MgCl_2$ showing loss of phase dense nucleoli. (C) After initial ionic conditions are restored, nucleoli rapidly reassembled and returned to their original shape, size, and position (×400).

of buffer A for 60–90 s. Coverslips were then washed with buffer A and incubated in the same buffer containing rabbit polyclonal antibody against Topo II (1/30; Topogen), human ascites against Topo I (1/300; Neovision), human ACA serum (1/300; gift from Dr. J. Rattner, University of Calgary), or mouse monoclonal antibody against Histone H1 (1/50; Biogenex). After washing, primary antibodies were visualized using secondary species-specific anti-IgG antibodies linked to fluorescein, biotin, or Texas Red (Amersham). Texas Red-linked avidin (Victor) was used to visualize the biotinylated secondary antibody. For chromatin staining, DNA binding dyes propidium iodide or DAPI (Sigma) were used at 0.2 $\mu$g/ml final concentration.

Microscopic images captured with a DAGE MTI camera were visualized and quantified using the integration program of BDS image analysis software (Oncor) on a Macintosh Quadra 800 computer. In certain studies, images were recorded using a 100× objective (1.3 N.A.; Nikon) in combination with a digital cooled CCD camera (Photometrics), and out-of-focus contributions were removed using the constrained iterative algorithm [22] from VayTek Microtome deconvolution software. For each deconvoluted image, five focal planes were recorded by stepping the microscope focal plane at 0.5-$\mu$m intervals. Immunolabeling was quantified by first defining the region of interest by tracking the mouse around the 2-D projection of the integrated signal. The mean intensity of staining in that area was then calculated within the linear range using IPLab quantitation software (Scanalytics).

## RESULTS

### *Ion-Induced Changes in Chromosome Structure in Living Cells*

We recently reported that chromosomes microsurgically removed from living mitotic cells and exposed to hypertonic salt concentrations disassemble beyond the limits of phase contrast resolution. This disassembly, which involves large-scale unraveling of DNA from its normally highly condensed form, reverses completely upon the return to the original salt concentrations [5]. We therefore set out to investigate whether changing ionic conditions would have the same effect on chromosomes inside the nuclei of living cells, where chromatin is separated from the extracellular medium by cellular and nuclear membranes.

Raising ionic strength in the culture medium from 0 to 65 mM $MgCl_2$, resulted in nearly immediate disassembly of the entire genome within mitotic cells, as previously observed with isolated chromosomes [5]. All of the phase dense chromosomes rapidly became phase lucent (Figs. 1A and 1B). This chromatin unfolding was reversible within less than 1 min after restoring the original ionic conditions (Fig. 1C). The same effect was observed with 410 mM NaCl. We then monitored cells with recondensed chromosomes for an additional hour to determine whether they remained functional, as measured by their ability to successfully complete mitosis. All of the chromosomes in salt-treated cells progressed normally through mitosis, resulting in the creation of two separate daughter cells (Figs. 1D–1F).

When exponentially growing interphase cells were exposed to the same ionic strength conditions, their nucleoli became diffuse and indistinct in less than 10 s demonstrating the rapid disrupting effect of ionic treatment on intranuclear structure (Figs. 2A and 2B). This effect reversed in less than 1 min by washing the cells free of high salts with the original cell culture medium (Fig. 2C). Quantitation of cell numbers and BrdU incorporation confirmed that incubation of interphase cells in medium with 65 mM $MgCl_2$ for 3 min did not have any significant effect on cell viability (data not shown). This finding is consistent with past studies which show that the effects of hypertonicity are fully reversible in interphase cells [23, 24]. Similar reversible changes in chromatin organization and nuclear morphology were also observed in mitotic and interphase cells upon treatment with 600 mM sucrose which does not cross the cell membrane, but not with 600 mM urea which rapidly enters cells and nuclei [25]. These results suggest that ionic alterations represent the primary force that drives changes in chromatin architecture in living cells exposed to high salts, rather than changes in osmolarity.

### *Ionic Sensitivity of Chromosomes in Detergent-Permeabilized Cells*

To confirm that the effects on chromosome structure that we observed resulted from changes in intracellular ion concentrations, we eliminated the lipid mem-

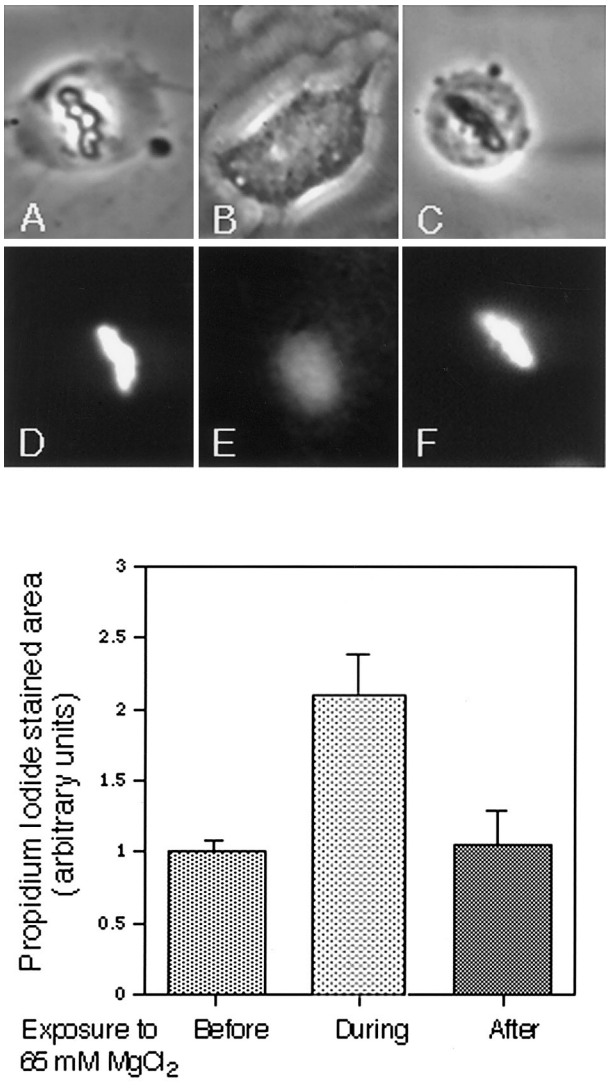


**FIG. 3.** Phase contrast (A–C) and fluorescence (D–F) images of propidium iodide-stained chromosomes in detergent-permeabilized metaphase cells. (A and D) Control cell extracted with 0.5% Triton X-100, before salt treatment. (B and E) Another mitotic cell during exposure to 65 mM $MgCl_2$ showing that chromosomes become phase lucent and DNA unravels over a wider region. (C and F) Salt-treated cell after return to the original buffer conditions, demonstrating recovery of normal condensed chromosome form (×600). Histogram: quantification of the area of DNA stained with propidium iodide in cells before, during, and after exposure to high salts.

brane barriers to added ions by permeabilizing the cells with the detergent Triton X-100 and incubated them in the physiologically relevant acetate buffer [21]. In these permeabilized cells, chromosomes underwent a similar cycle of disassembly–reassembly in response to varying salt concentrations, as previously observed in living cells (Fig. 3 versus Figs. 1A–1C). Permeabilized metaphase cells were then incubated with the DNA-binding dye propidium iodide to study the quantitative and qualitative aspects of the salt-induced chromatin unfolding in more detail (Fig. 3). Because DNA-binding dyes change the mechanical properties of chromatin [5], we used different cells for staining of condensed, unfolded, and recondensed chromosomes. The results of these experiments show that the chromosome region stained with the DNA-binding dye expanded by 200% inside mitotic cells during exposure to high salt (Figs. 3B and 3E versus Figs. 3A and 3D) and then spontaneously condensed back to its original dimensions when the salt concentration was lowered (Figs. 3C and 3F). In contrast, sucrose had no visible effect on chromatin structure once membrane barriers were compromised, again suggesting that the observed effects were due to changes in ion concentration and not in osmolarity.

Although recondensation appeared to be complete when viewed at the level of the whole genome, it was possible that chromosome fine structure was lost. We therefore examined the structural integrity of the kinetochore domains of the centromere regions that are known to mediate chromosome attachment to the mitotic spindle [26, 27]. When we used ACA antibodies [27, 28] to stain these kinetochore domains within chromosomes that were induced to disassemble and reassemble by varying salt conditions, we observed patterns of staining in recondensed chromosomes that were similar to those of controls (Fig. 4). These results suggest that the subchromosomal organization is faithfully retained in these regions after ion-induced reassembly *in situ*.

### *Effects of Ionic Conditions on Chromatin Composition*

Proteins that are extracted into the soluble phase in response to salt treatment of chromosomes in permeabilized cells should be diluted greatly as they diffuse into the surrounding culture medium and are removed in subsequent wash steps. Thus, it is unlikely that these extractable proteins are required for the chromosome reassembly that we observed when normal salt conditions were restored. Therefore, we set out to determine whether chromatin-associated proteins that have been suggested to be important for chromosome structure, such as Topo I [9], Topo II [10, 13], and histone H1 [6, 8], are resistant to high-salt extraction *in situ*.

Quantitation of immunofluorescence labeling in permeabilized mitotic cells revealed that at least 25% of Topo II remains associated with chromatin in these cells after extraction with high salt (magnesium or sodium; Figs. 5A–5H). However, the decrease of Topo II immunostaining in salt-treated cells (Fig. 5G) also could be due to the decreased accessibility of Topo II protein to antibodies following the chromosome reassembly. Therefore, we preincubated cells with the nonhydrolyzable $\beta,\gamma$-imido analogue of ATP (AMP-PNP)

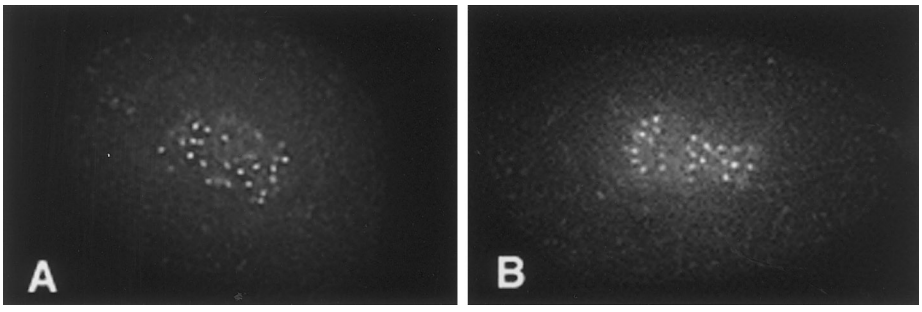


**FIG. 4.** Kinetochore labeling with ACA antibodies in (A) control (not treated) and (B) NaCl (410 mM)-treated mitotic cells (×1200). The punctate pattern of kinetochore staining remained essentially unchanged after ion-induced chromosome disassembly and reassembly.

before exposure to high salts (Figs. 5I–5M). AMP-PNP has been shown to trap Topo II in a stable, salt-resistant complex with plasmid DNA during the last stage of its catalytic cycle *in vitro* [29, 30]. In our system, AMP-PNP did not interfere with the salt-induced chromatin unfolding–refolding cycle. However, the intensity of immunostaining for Topo II in cells preincubated with AMP-PNP was comparable with the control, presumably because this ATP analogue stabilized the Topo II–DNA bond, thereby making it resistant to salt extraction (Figs. 5I–5M). This result suggests that in the absence of AMP-PNP, one pool of Topo II encompassing about 70–75% of the total Topo II protein is indeed salt-soluble.

The images of Topo II immunostaining were collected using standard epifluorescence microscopy and thus may contain an undefined amount of out-of-focus noise. Therefore, we analyzed the ability of Topo II, Topo I, and Histone H1 to resist salt extraction using digitized deconvolution confocal microscopy to acquire multiple optical slices within individual cells and to remove out-of-focus contributions. Quantitative analysis of the dehazed images produced results for chromatin-associated Topo II (Figs. 6A–6F) that were nearly identical to those obtained in our previous measurements (26% versus 25%; Fig. 5). We also found that chromatin of salt-treated cells conserve more than 50% of Histone H1 (Figs. 6G–6J). In contrast, more than 95% of Topo I (Figs. 6K–6N) was extracted from recondensed chromosomes of permeabilized cells. Thus, in contrast to Topo II and Histone H1, Topo I does not appear to be critical for chromosome remodeling in these cells.

## DISCUSSION

The effects of varying ionic strength on chromatin structure have been studied in the past by a wide range of techniques, including sucrose gradient sedimentation, circular dichroism, pulse field electrophoresis, and electron microscopy [23, 24, 31–33]. These approaches often include harsh treatments such as DNase digestion, baking, and fixation with alcohols or aldehydes, all of which irreversibly alter the dynamic properties of chromosomes. Therefore, although the ionic microenvironment has been previously proposed to be important for control of chromatin architecture [for review see 34], little information is available about its effects on higher order chromatin organization and dynamics *in vivo*. Here, we used nondisruptive techniques of phase and fluorescence microscopy to describe structural remodeling within chromosomes *in situ* induced by changes in ionic conditions in living and permeabilized cells in the absence of fixation.

Our results unveil the ability of chromosomes to reversibly change their higher order conformation inside cells in response to variations in the ionic environment. We propose that in condensed mitotic chromatin, the repulsion forces resulting from the abundance of charged phosphate groups in the DNA are balanced by attractive forces generated by electrostatic interactions between chromatin-bound proteins. When salts are added, these electrostatic interactions are cancelled, repulsion forces prevail, and chromatin structure is shifted towards a more unfolded form. After washing salts away, the "structural memory" templated in deeper levels of chromatin organization allows reformation of disrupted protein–protein bonds, with precision sufficient for cell survival. In fact, intact cells that underwent reversible disassembly and reassembly of their chromosomes *in situ* in response to raising and then lower ionic concentrations, commonly continued on to undergo normal cell division. This model also could account for the reversible morphological changes observed in living interphase cells exposed to varying ionic concentrations. These results are in agreement with earlier reports showing that inhibition of cell cycle induced by hypertonicity in the culture medium is reversible upon restoration of optimal cell culture conditions [23, 24, 35].

The method for analysis of chromosome architecture *in situ* was then used to address the question of which chromatin proteins could be required for maintenance

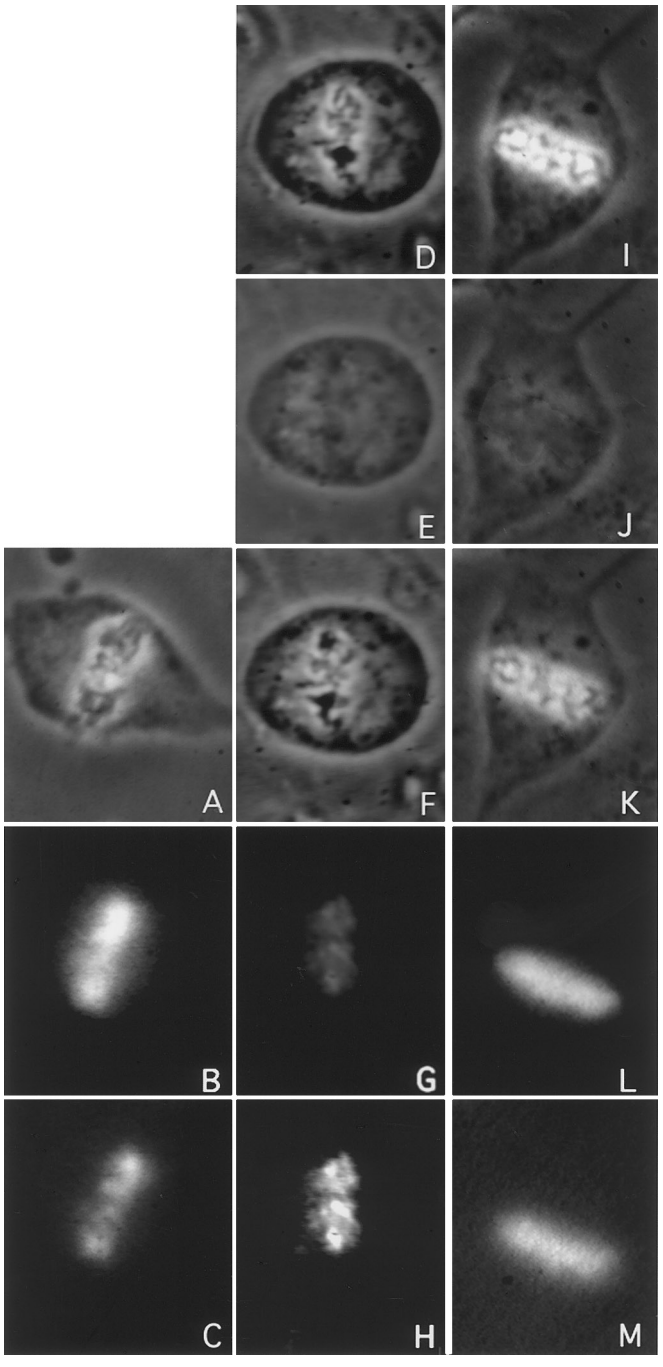


**FIG. 5.** Effect of high salt on association of Topo II with chromatin in detergent-permeabilized metaphase cells. Phase contrast (A, D–F, I–K) or fluorescence views showing cells stained with anti-Topo II$\alpha$ antibody (B, G, L) or DAPI (C, H, M) to visualize DNA. (A–C) Control metaphase cell. A metaphase cell before (D), during (E) and after (F–H) exposure to 65 mM $MgCl_2$. (I–M) A metaphase cell preincubated with AMP-PNP before (I), during (J) and after (K–M) treatment with 65 mM $MgCl_2$ ($\times$1000).

of condensed chromosome structure. We examined three chromatin-associated proteins, Topo I, Topo II, and Histone H1, within ion-disassembled and reassembled mitotic chromosomes. The finding that a pool of Topo II stays associated with chromosomes after chromatin unfolding is consistent with a model in which DNA-associated Topo II plays a structural role in higher order chromosome organization [13, 14]. In this model, Topo II binds simultanously to DNA loops and to the insoluble chromosome backbone (the nuclear scaffold, [36]), thereby acting as a DNA loop fastener. The presence of a "structural" pool of Topo II in mitotic chromosomes is also supported by the finding in human A431 epidermoid cells that the amount of chromatin-bound Topo II is higher during mitosis, although its catalytic activity is lower [10]. Our results also suggest that mammalian chromosomes might be structured differently than chromosomes condensed from sperm DNA in *Xenopus* egg mitotic extract where full Topo II extraction can be achieved by a mild salt treatment [18].

In the present study, we also demonstrated that AMP-PNP acts as a "biological fixative" [37] *in situ* by stabilizing the association of Topo II with native chromatin. This suggests that Topo II remains bound to mitotic chromosomes in a catalytically relevant manner in our system. The stabilization of this bond did not, however, prevent ion-induced reversible chromosome unfolding. Thus, the process of ion-driven chromosome unfolding does not appear to require the disruption of Topo II–DNA binding interactions. Moreover, the level of Topo II fluorescence remained nearly constant in AMP-PNP-treated chromosomes after ionic treatments. This finding provides direct evidence to suggest that the levels of fluorescence staining measured in native chromosomes reflected changes in the actual level of bound protein, rather than alterations in their accessibility to antibodies following recondensation. This is further supported by the finding that the kinetochore domains remained unaffected by ions in our system.

Our finding that Histone H1 is partially resistant to solubilization by ionic treatment is in agreement with the well known role of this protein in chromatin fiber organization [31, 38] and its proposed function in higher order chromosome architecture [8, 39]. Accordingly, it is possible that the salt-induced chromosome decondensation results from the disruption of electrostatic bonds between histone H1 and nucleosomes. This disruption could result from rearrangements in overall nucleosome structure, which have been shown to occur in response to varying ionic conditions [40]. The view above might be controversial in the light of more recent reports showing that Histone H1 is not essential for chromosome condensation and cell survival in certain cell types [19, 41]. However, redundancy often provides stability, and in the case when Histone H1 is missing, its function may be compensated by another set of proteins in the cell. For example, cut3 gene

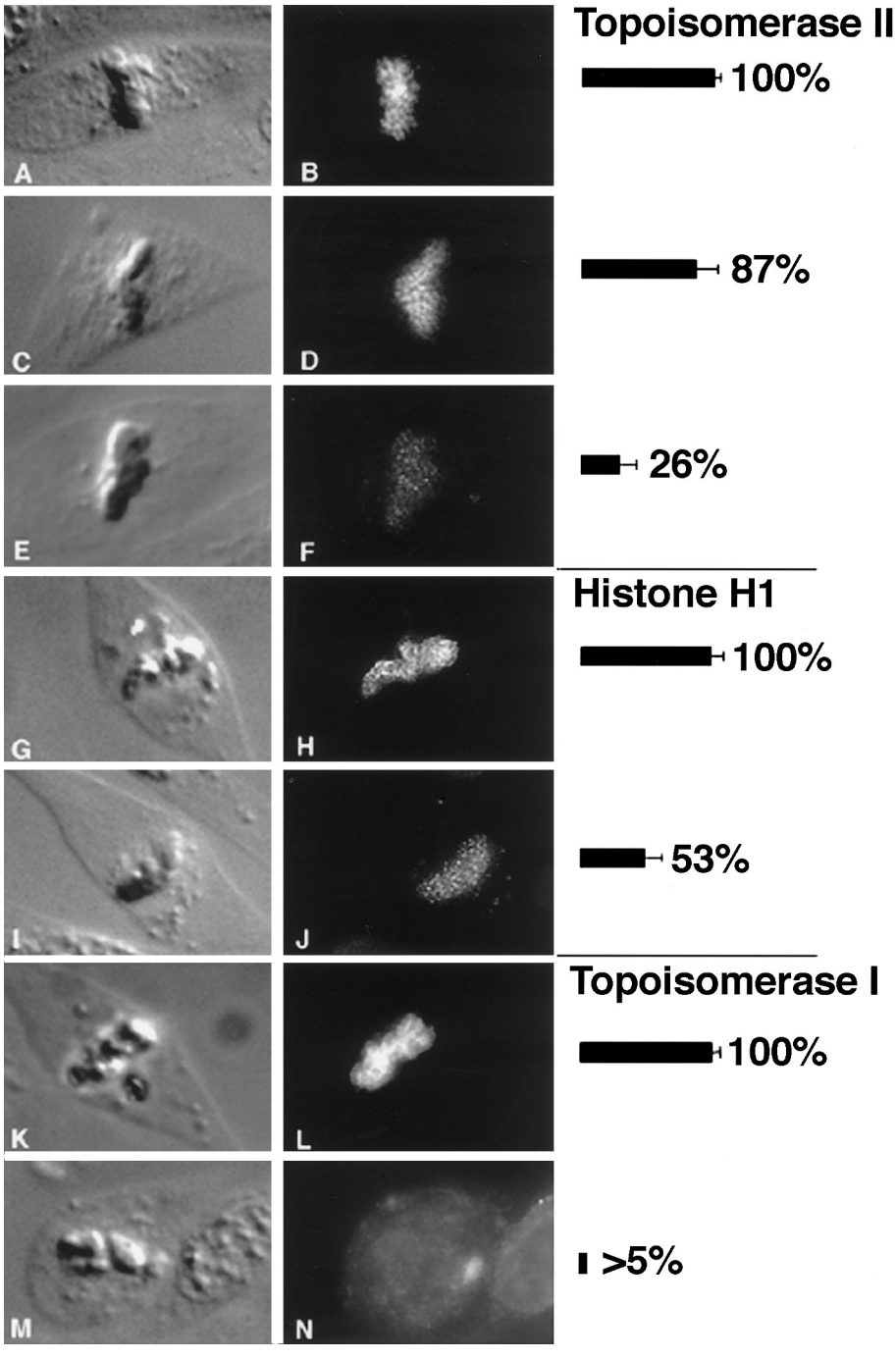


**FIG. 6.** Differential interference (left) and deconvoluted immunofluorescence (right) microscopic images showing association of Topo II (A–F), Histone H1 (G–J), and Topo I (K–N) with mitotic chromosomes in control (A, B, G, H, K, L) and $MgCl_2$-treated (C–F, I, J, M, N) cells (×850). (C and D) a cell labeled with anti-Topo II antibody which was preincubated with AMP-PNP before salt treatment. The image in (N) showing salt-treated cell stained with anti-Topo I antibody is overexposed to clarify the lack of positive signal. Black bars represent the percentages of immunofluorescence staining in ion-treated cells relative to controls; each bar represents the average measurement obtained from 20 different cells in two independent experiments.

mutation in yeast which affects chromatin condensation during mitosis, can be partially complemented by overexpression of Topo I [42]. In contrast to Topo II and Histone H1, chromatin-associated Topo I was totally extracted during the ion-induced decondensation–recondensation cycle. Therefore, we conclude that the presence of Topo I on metaphase chromosomes is not needed to maintain their condensed shape under nor-

mal, physiological conditions. Nevertheless, we cannot exclude its participation at a more subtle structural level, not detectable in our system.

In summary, we have described a new model system for studying the effects of ionic conditions on intact chromosomes in living and detergent-permeabilized cells. This system was then used to screen for proteins that potentially may be important for maintenance of condensed chromosome form. Because this system permits analysis of chromosome dynamics *in situ*, it may facilitate future studies that focus on the molecular and biophysical basis of chromosome structure and dynamics.

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