

Reversible Hypercondensation and Decondensation of Mitotic Chromosomes Studied Using Combined Chemical–Micromechanical Techniques

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Abstract We show that the chromatin in mitotic chromosomes can be drastically overcompacted or unfolded by temporary shifts in ion concentrations. By locally ‘microspraying’ reactants from micron-size pipettes, while simultaneously monitoring the size of and tension in single chromosomes, we are able to quantitatively study the dynamics of these reactions. The tension in a chromosome is monitored through observation and calibration of bending of the glass pipettes used to manipulate the chromosomes. For concentrations > 500 mM of NaCl and > 200 mM of MgCl₂, we find that the initially applied tensions of ~500 pN relax to zero and that mitotic chromatin temporarily disperses in agreement with previous work (Maniotis et al. [1997] *J. Cell. Biochem.* 65:114–130). This unfolding occurs in about 1 s, and is reversible once the charge density is returned to physiological levels, if the exposure is not longer than ~1 min. Low concentrations of NaCl (< 30 mM) also induces a decrease in tension and increase in size. We observe this swelling to be isotropic in experiments on chromosomes under zero tension, a behavior inconsistent with the existence of a well-defined central chromosome ‘scaffold’. By contrast 10 mM of divalent cations (MgCl₂ and CaCl₂) induces an extremely rapid and reversible increase in tension and a reduction in the size of mitotic chromosomes. Hexaminecobalt trichloride (trivalent cation) has the same effect as MgCl₂ and CaCl₂, except the magnitude of force increase and size change are much larger. Hexaminecobalt trichloride reduces mitotic chromosomes to 65% of their original volume, indicating that at least 1/3 of their apparent volume is aqueous solution. These results indicate that chromatin inside mitotic chromatids has a large amount of conformational freedom allowing dynamic unfolding and refolding and that charge interactions play a central role in maintaining mitotic chromosome structure. *J. Cell. Biochem.* 85: 422–434, 2002. © 2002 Wiley-Liss, Inc.

Key words: mitotic chromosomes; chromatin; electrostatics; DNA-protein interaction

Chromosome structure and its modulation are critical to all aspects of cell function. During interphase, control of chromosome structure at the chromatin level is important for the success

and regulation of transcription, replication, and recombination [Widom, 1997]. As the cell enters mitosis, larger-scale chromosome structure becomes central to disentanglement and compaction of the two chromosome copies, in order for successful segregation of chromosomes to the daughter cells to occur [Heck, 1997]. Chromosome structures at these different periods of the cell cycle are intensely studied, yet there is not a clear picture of large-scale structure of either interphase or mitotic chromosomes [Koshland and Strunnikov, 1996; Widom, 1998].

A powerful tool used to study chromosome (and particularly chromatin) structure is its perturbation by varying ionic conditions. The reason for the importance of electrostatic interactions to chromatin structure is simply that DNA itself is highly negatively charged, and the attractive part of histone–DNA interactions is

Grant sponsor: Whitaker Foundation (Biomedical Engineering Research Grant); Grant sponsor: National Science Foundation; Grant number: DMR-9734178; Grant sponsor: Research Corporation (Research Innovation Grant); Grant sponsor: Petroleum Research Foundation of the American Chemical Society (AC Grant); Grant sponsor: Johnson and Johnson (Focused Giving Grant).

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Received 22 August 2001; Accepted 8 January 2002

DOI 10.1002/jcb.10132

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largely due to electrostatic attraction. Changes in univalent ion concentration change the range of electrostatic interactions, and can be used to 'tune' their strength. Di- and multivalent ions can also do this, but at relatively low concentration can also mediate *attractive* interactions between like-charged macromolecules [Gelbart et al., 2000].

In this article, we will examine how these kinds of effects occur on whole mitotic chromosomes, using an experimental technique that allows the dynamics of changes in mechanical properties (i.e., degree of compaction and extensibility) to be monitored. Our motivation is to use the ionic strength shifts to modulate nucleosome–nucleosome interactions, and to study how overall mitotic chromosome structure depends on those interactions.

The effect of changing ionic conditions on chromatin structure and nucleosome–nucleosome interactions has been widely studied. Increasing the concentration of NaCl above 500 mM causes the successive disassociation of histones from the chromatin fiber [van Holde, 1988], due to the reduction in strength of electrostatic interactions that occurs at high univalent ionic strength. Lowering the concentration of NaCl to 10 mM converts the 30 nm-chromatin fiber to the 10-nm 'beads on a string' chromatin fiber; thanks to the electrostatic repulsion of adjacent nucleosomes that occurs at low univalent ionic strength. However, addition of ~ 1 mM of $MgCl_2$ maintains the 30-nm fiber at 10-mM NaCl [Ausio et al., 1984; Zlatanova et al., 1998]; thanks to attractive nucleosome–nucleosome interactions mediated by the divalent ions. Above ~ 1 -mM $MgCl_2$, chromatin fibers begin to aggregate and stick to each other [Borochoy et al., 1984], again due to divalent-cation-induced attractive interactions. Higher valence ions such as hexaminecobalt trichloride (a trivalent cation) show this ability to compact chromatin and cause aggregation at lower concentrations than divalent ions [Sen and Crothers, 1986; Smirnov et al., 1988].

Since variation in ionic conditions change nucleosome–nucleosome interactions and the structure of isolated chromatin fibers in micromanipulation experiments, the elastic properties of chromatin should also be affected. Cui and Bustamante [2000] studied the elasticity of isolated chromatin fibers in micromanipulation experiments, and showed how various concentrations of NaCl affected their elastic response.

They found that at 5 mM NaCl, chromatin fibers adopt an extended 10-nm-fiber-like form, which deforms continuously from its random-coil initial state, with a positive curvature in the force–extension response rather similar to that of dsDNA. In this low ionic strength regime, chromatin fiber was observed to behave rather like a flexible polymer, showing no tendency for nucleosomal aggregation. At higher concentrations of 40 and 150 mM NaCl, the chromatin fiber was seen to exhibit a transition 'plateau' in its force–extension response, which could be understood as mechanically-driven unfolding of the initially condensed 30-nm fiber, to the uncondensed 10-nm fiber. The results of those experiments are thus in good accord with previous biochemical and biophysical experiments on isolated chromatin fibers at low and physiological salt concentrations.

Changes in ionic conditions have also been observed to affect the cellular organization of chromatin fibers, in interphase nuclei and in mitotic chromosomes. The effects of KCl and $MgCl_2$ on interphase chromosomes in isolated nuclei are consistent with studies of ionic effects on isolated chromatin fibers [Aaronson and Woo, 1981]. Inside isolated nuclei, chromatin is the most condensed at about 200 mM KCl, and gradually disperses for concentrations above and below 200 mM. In contrast, the same authors observed that $MgCl_2$ induces a sharp compaction at 1 mM, and the chromatin stays compacted until about 50 mM where the chromatin gradually disperses again.

One of the best-known experiments using ions to perturb chromosome structure was use of 1 M NaCl to first histone-deplete metaphase chromosomes, which were then observed with the electron microscope [Paulson and Laemmli, 1977]. Loops of about 80 kb of DNA were observed emanating from a dense protein core, leading to the proposal of a scaffold model for mitotic chromosome structure. This scaffold was also found to undergo a lateral aggregation in the presence of 5 mM $MgCl_2$ [Earnshaw and Laemmli, 1983]. Although intuitively appealing, the interpretation of these experiments depended on the assumption that the histone-depletion reaction and subsequent preparation for electron microscopy did not substantially alter the proposed scaffold organization.

A recent experiment [Maniotis et al., 1997] pursued an approach similar to that of Paulson and Laemmli [1977], but while observing the

chromosomes in physiological buffer using light microscopy. Maniotis et al. [1997] showed that mitotic chromosomes extracted from cells using glass needles could be made to rapidly decondense and recondense when either 500 mM MgCl₂ or 1 M NaCl was pipetted into the cell culture dish. Similar results were found for chromosomes *in vivo*, where extracellular addition of 410 mM NaCl or 65 mM MgCl₂ caused cycles of decondensation–condensation of the mitotic chromosomes *inside* bovine capillary endothelial cells [Bojanowski and Ingber, 1998].

The results of the experiments of Maniotis et al. [1997] and Bojanowski and Ingber [1998] suggest that temporary exposure of mitotic chromosomes to strong shifts in ionic strength can cause large-scale unfolding of chromosomes, which is apparently reversible (i.e., the mitotic chromosomes were observed to regain a native-like conformation). We were motivated to carry out experiments, which combined the basic idea of the studies of Maniotis et al. [1997] and Bojanowski and Ingber [1998], with our ability to study chromosome elastic response [Poirier et al., 2000] to quantitatively answer the following questions:

- (1) How does the degree of decondensation (assayed using changes in visible size and elastic response) of mitotic chromosomes vary with ionic strength, and with valence of the cations used?
- (2) What is the timescale for ionic-strength chromosome decondensation to occur (assayed through dynamic measurement of tension changes in a chromosome)?
- (3) How reversible are ionic-strength decondensation–condensation cycles (assayed using measurements of elasticity before and after ionic strength exposures)?
- (4) How isotropic is ionic-strength-mediated decondensation, and can any evidence of an internal ‘scaffold’ structure (e.g., a directly observable structure, or a lower limit on the degree to which chromosome elastic response is reduced by ion exposure) be observed?

We thus conducted experiments on single mitotic chromosomes removed from newt (*Notophthalmus viridescens*) epithelial cells with micropipettes during the early stages of metaphase. Newt chromosomes are well suited to micromanipulation experiments, and have

had their basic elastic properties well quantified previously [Poirier et al., 2000, 2001]. In most of the experiments of this article, isolated chromosomes were stretched to an initial force sufficient to cause a ~50% elongation, and then were microsprayed with different ion types and concentrations. During spraying, we monitor both the force the chromosome supported, (by observation and later force calibration of pipette bending) and chromosome size, by acquisition of a series of light microscope pictures.

We find, in agreement with Maniotis et al. [1997] that high concentrations of either NaCl or MgCl₂ induce dispersion of the chromosome, into a ‘cloud’ of chromatin fibers much larger than the initial chromosome, and with a much lower contrast in the phase microscope. For sufficiently large ion concentrations, the tension supported by the chromosome can be reduced to zero. Associated with this increase in size is a decrease in the tension that the chromosome initially supported. The dynamics of these reactions are very rapid; decondensation occurs in a few seconds and the recondensation occurs in less than a second. In some cases, we observe that the tension in the chromosome is able to change faster than we are able to locally shift the ionic conditions. Remarkably, as long as the shifts away from physiological ionic conditions were shorter than ~1 min in duration, the chromosome refolds into a structure with nearly native morphology and elastic properties. Although not the main focus of this study, we did observe that exposures to high (~1 M) NaCl concentrations eventually irreversibly change chromosome shape and elasticity, as expected based on, for example, solution-phase studies of removal of histones from chromatin after long-duration exposures to high NaCl concentrations [van Holde, 1988].

Effects of lower salt concentrations on chromosome elasticity depend on the valence of the ion used. Thirty millimolar NaCl induces a drop in the measured force and density in a few seconds. Once the chromosome is returned to physiological conditions, the force returns to the original value in about 1 s; 10 mM MgCl₂ induces the opposite effect. There is an increase in the force and the volume decreases in about 1 s. Hexaminecobalt trichloride has a similar, but more pronounced effect to MgCl₂, causing a five-fold increase in the force and reducing the chromosome to 2/3 of its original volume.

Thus, in general, we have found that low concentrations of multivalent ions can induce hypercondensation of mitotic chromosomes.

Our results show that shifts in ionic strength can cause the chromosome's internal structure, which supports an applied force, to be completely disrupted. In the case of low concentrations of di- or trivalent cations, chromosomes become hypercondensed and become elastically stronger. These effects are in close correlation with effects observed for chromatin fibers and lead us to the conclusion that the main cause of the large-scale effects that we observe is modulation of the electrostatic portion of nucleosome–nucleosome interactions. Thus, charge interactions play a crucial role in maintaining large-scale mitotic chromosome structure.

A number of general observations come out of our experiments as well. The rapidity with which we can unfold and refold chromosomes indicates that the chromatin in a metaphase chromatid has a large amount of conformational freedom allowing its dynamic unfolding and refolding. Remarkably, this unfolding and refolding does not lead to large changes in chromosome elasticity, which we argue indicates that the initial native state does not have a highly ordered structure (see Discussion). We find that 1/3 of the volume of a native metaphase chromosome can be rapidly 'squeezed out', again indicating a relatively loose native organization of chromatin fibers.

Finally, we find no evidence for a mechanically solid internal mitotic 'scaffold'. Ion-driven chromosome decondensation is essentially isotropic (inconsistent with a loops-hanging-from-scaffold picture). Furthermore, when electrostatic interactions are screened away, the chromosome completely unfolds, leaving no mechanically solid structure. The general behavior of the mitotic chromosome is, therefore, essentially that of a crosslinked network of chromatin fibers, with no global organization of the crosslinking elements.

MATERIALS AND METHODS

Cell Culture

TVI newt (*N. viridescens*) epithelial cells [Reese, 1979] were grown in 75-ml cell culture flasks (Falcon) in 10 ml of cell culture medium [Poirier et al., 2000]. The culture medium was replaced every 4 days, and at 90% confluency, the cells were subcultured into new flasks with

0.15% trypsin in HBSS (Cellgro). Experiments were done in custom-made culture dishes with a diameter of 20 mm and a depth of 2 mm. These dishes are made of two teflon rings, which are stacked on a 40 mm diameter #1 cover slide and attached by paraffin. The cells were subcultured into 1.8 ml of culture medium and grown to about 70% confluency in the small dishes. Micromanipulation experiments were done in these dishes, while the cells were between 70 and 100% confluent. Growing the cultures to a point where adjacent cells provided mechanical support to one another was important since this allowed the forces necessary for chromosome removal to be applied to mitotic cells without dislodging them.

Experimental Setup

Chromosomes were imaged by an inverted light microscope (IX-70 Olympus) with a 60 \times , 1.4 NA objective using either phase contrast or differential interference contrast (DIC) imaging (Fig. 1). The culture dish was positioned by a three-axis focus control stage (Prior Scientific).

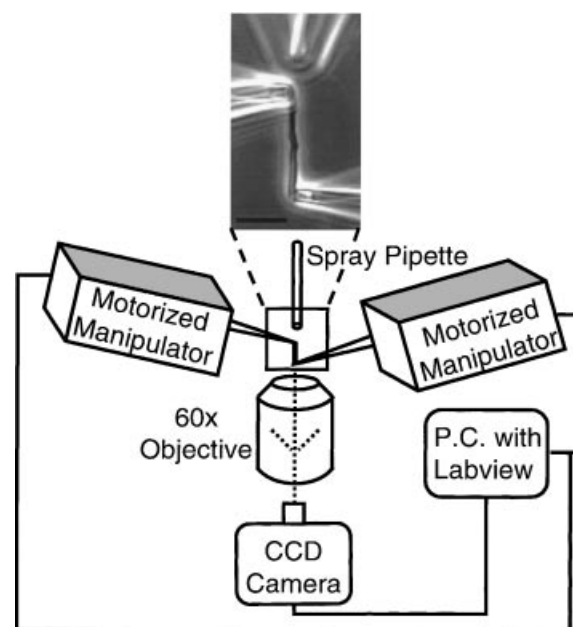


Fig. 1. Experiments were done on an inverted microscope in cell culture buffer. Two pipettes, positioned by motorized computer controlled manipulators, hold the chromosome for chemical–micromechanical experiments. A third pipette, positioned close to the chromosome, is used to flow in a buffered salt solution. Forces are measured by observing the deflection of the bottom pipette. A PC with Labview and Imaq acquires images at 10 Hz before, during, and after the chromosome is exposed to the salt solution, allowing for continuous force measurement.

Chromosome extraction and manipulation were done with two motorized XYZ micromanipulators (MP-285 Sutter) mounted on opposite sides of the microscope. Ionic conditions were changed by spraying with a third pipette held and positioned by a manual micromanipulator (Taurus, WPI). A 233-MHz Pentium PC with Labview (National Instruments) was used to control the micromanipulators and image acquisition. The images were recorded by a CCD video camera (Panasonic) and captured with an NI-IMAQ PCI-1408 card and NI-IMAQ (National Instruments) software onto a PC.

Chromosome Extraction

Two micropipettes, each attached to a micromanipulator, were used to isolate a mitotic chromosome. Micropipettes with an inner diameter of 2 μm and a bending stiffness of about 10 nN/ μm were fabricated from borosilicate glass pipettes (WPI) as described in Poirier et al. [2000]. One pipette is filled with 0.05% Triton-X (FisherBiotech) in 60% PBS (Biowhitaker) while a second pipette is filled with 60% PBS. After a cell between prometaphase and metaphase is located, the first pipette is positioned within a few microns of the edge of the mitotic cell. The Triton-PBS solution is then flowed out of the cell with 5,000 Pa of pressure. After 30–60 s a 5–10 μm hole in the cell membrane appears, and the chromosomes flow out of the cell. Usually, the chromosomes are well attached to each other and the cell, but in about 1 in 100 attempts, a chromosome floats completely free. When this occurs, the second pipette is positioned within 1 μm of the end of the free chromosome, and is aspirated into the pipette with about 50 Pa of pressure. The chromosome tip permanently adheres to the inner wall of the pipette after 30–60 s of contact, via non-specific adhesion of chromatin to untreated glass. The cell is then moved away from the isolated chromosome and the chromosome is positioned about 40 μm above the glass surface.

More often, a chromosome does not float completely free, but is connected to second chromosome by a thin fiber, which were observed by Maniotis et al. [1997]. The second pipette aspirates the free end, but the chromosome is still anchored to the cell. These attachments tend to be end to end, and extend the chromosome when the cell is moved away. Also, the attachments do not break until the chromo-

some becomes extended beyond five times, which irreversibly damages the chromosome [Poirier et al., 2000]. To avoid this, a third pipette is used to aspirate the end of the chromosome anchored by the thin fiber. (This pipette is made relatively flexible because it will be used to measure forces as described in the next section.) The cell is moved away and the third pipette instead of the chromosome now supports the force applied to the thin fiber. The thin fiber then breaks without damaging the chromosome. (A movie of this procedure is available at <http://safarsquid.phy.uic.edu/~mpoirier/experiments/movies/chromo-extract.mpg>.)

Elasticity Experiments

To quantify the chromosome force constant (the force needed to double its length), a third pipette is fabricated with an inner diameter of 2 μm and a force constant of about 0.5 nN/ μm . (1 nN = 1 nanonewton = 10^{-9} newton; the force constant indicates the perpendicular force that should be applied at a pipette tip to deflect it a given distance, thus a 0.5 nN/ μm pipette will be deflected 2 μm by a 1 nN force) This pipette is positioned within 1 μm of the free end of the isolated chromosome, which is then aspirated with 50 Pa of pressure. The pipettes are then positioned anti-parallel to each other and perpendicular to the chromosome (Fig. 1). The PC with Labview then moves one pipette out and back at a strain rate of 0.01 s^{-1} (strain rate is the inverse of the time taken to double the chromosome's length), periodically capturing images to disk. The bending deflection of the stationary pipette provides a measure of the force applied to the chromosome during its extension. (A movie of typical extension–retraction cycles showing pipette bending is available at <http://safarsquid.phy.uic.edu/~mpoirier/experiments/movies/chromo-extend.mpg>.)

Following an experiment, the images were analyzed to measure length of the chromosome as a function of deflection of the stationary pipette. Pipette deflection is converted to a force by measuring the force constant of the stationary pipette by pushing it against a previously calibrated pipette [Poirier et al., 2000] after the experiment. The force constant of the chromosome f_0 (the force needed to stretch the chromosome to double its native length) is determined from the slope of the force vs. extension plot and can be converted to a Young (stretching)

modulus [Landau and Lifshitz, 1986] by dividing it by the cross-sectional area of the chromosome, i.e., $4 \mu\text{m}^2$.

Previous experiments on primary cultures of newt lung epithelia [Poirier et al., 2000] showed that the force constant of newt chromosomes vary over a small range from 0.5 to 1 nN, and that for stretching to about four times native length, the stretching force grows linearly with elongation. The TVI newt cells of this study have the same native elastic properties [Poirier et al., 2001].

Salt Experiments

The effect of ionic concentration was studied by locally spraying a chromosome held in the two pipettes (Fig. 2). A spray pipette was fabricated as described above and then filled with 50 mM Tris-HCl plus various concentrations of NaCl, MgCl₂, CaCl₂, or Co(NH₃)₆Cl₃. A third manual manipulator (Taurus, WPI) was used to position the spray pipette between 10

and 70 μm of the chromosome. The chromosome was then stretched, and the force was monitored as described above. After some tension is applied to the chromosome, a 40 s time series of pictures was acquired at 10 frames/s by Labview with IMAQ. Five seconds into the time series, the salt solution was sprayed for 10 s. This was repeated on the same chromosome with increasing salt concentrations by varying the distance of the chromosome from the pipette. Determination of the salt concentration as a function of position from the pipette is described below.

Microspray Concentration Calibration

The concentration of ions decreases as one moves away from the pipette. To calibrate the concentration as a function of distance from the pipette, we used fluorescence of ethidium bromide, which has a diffusivity similar to the salts used in this study. Pipettes with diameters of 1.4, 1.6, 1.8, 2.5, 2.6, 2.8, and 3.1 were fabricated and filled with 0.3 mM ethidium

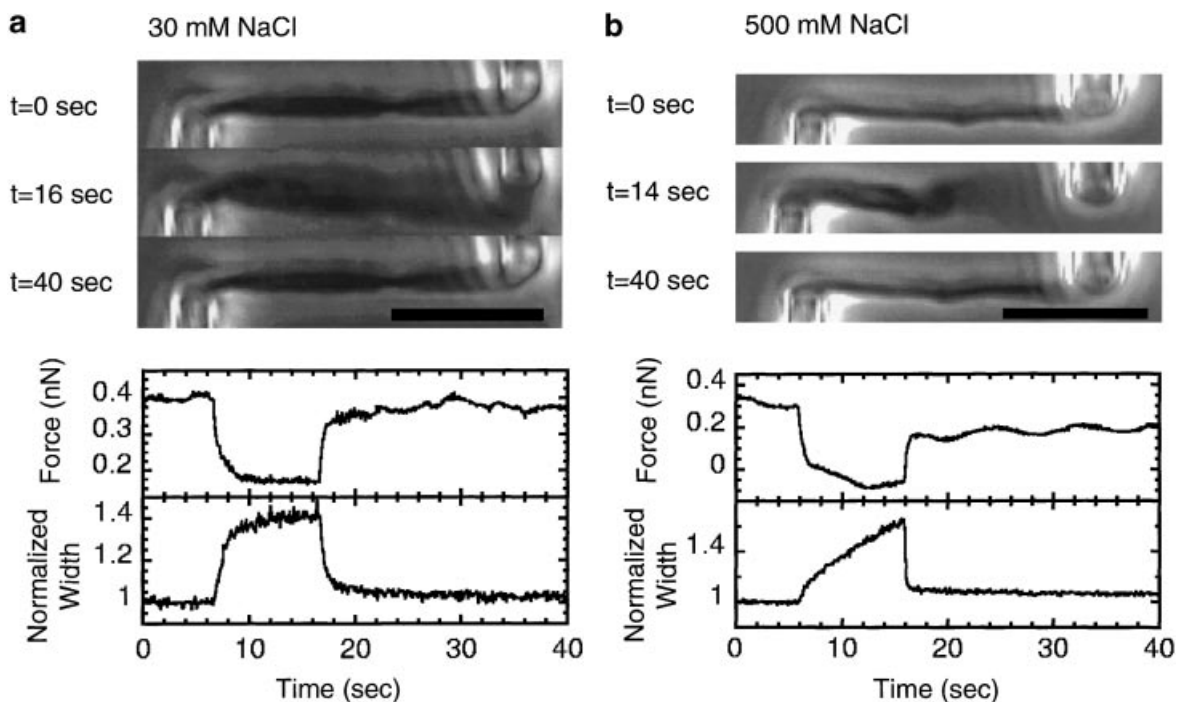


Fig. 2. Images of combined chemical–micromechanical experiments with 30 mM NaCl (a), 500 mM NaCl (b), 20 mM MgCl₂ (c), 300 mM MgCl₂ (d), and 40 mM Co(NH₃)₆Cl₃ (e). The images show the chromosome before, during, and after an exposure to the different ionic conditions. The plots show the time series of the force the chromosome supports and width of the chromosome. For 30 and 500 mM NaCl, the force decreases and the width increases. However, 20 mM MgCl₂ induces an increase in the force and decrease in width. 300 mM MgCl₂

causes a decrease in force and increase in width. Also, as the MgCl₂ is increased to 300 mM through 20 mM, there is a transient increase in the force. Forty millimolar Co(NH₃)₆Cl₃ is similar to MgCl₂ in terms of the qualitative response of the chromosomes, however, the magnitude of the change in force and width are about five times larger. The response of the chromosome to the ionic shifts occurs on the second time scale, and shows that the internal structure of a mitotic chromosome can be rapidly changed. Bar = 10 μm .

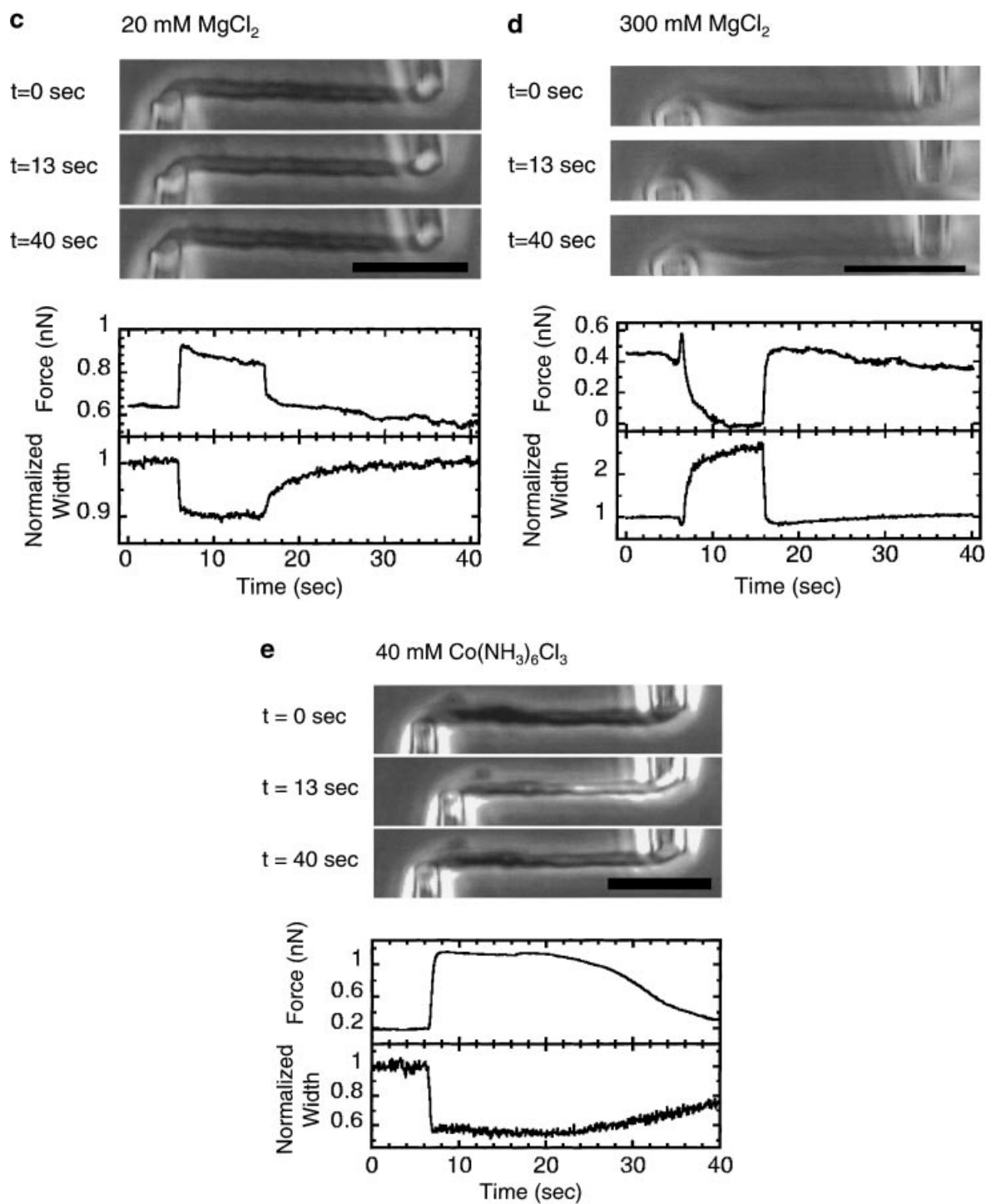


Fig. 2. (Continued)

bromide diluted in PBS. Forty second time series of images were acquired for 10 s sprays of ethidium bromide, with fluorescence excitation turned on about 5 s before spraying. Four time series were acquired for each pipette,

where two were with 25% excitation intensity and two were with 10% excitation intensity. The intensity decreased by about 2.5 times for the time series with 10% excitation light. This shows the CCD camera is linear over the

intensity range we are using. Also, the fluorescence intensity was observed to be similar for pipettes with similar diameter.

The spray pattern observed in fluorescence was always predominantly forward, with the fluorescence typically opening up with a $\sim 45^\circ$ angle. A plot of fluorescence intensity vs. distance from a pipette with a diameter similar to the one used in a given experiment was used as a calibration curve. This calibration is rough since out-of-focus light contributes to the measured pixel intensity. For wide pipettes ($3\ \mu\text{m}$), it is, therefore, possible for the observed fluorescence intensity to rise slightly as one goes away from the pipette opening. In this case, the fluorescent intensity initially increases to a maximum of double the intensity at the tip of the pipette $50\ \mu\text{m}$ from the tip. This occurs because the concentration initially decreases slowly enough that the increasing volume over which the fluorescent molecules are spread causes the apparent intensity to increase. From this, we estimate the error of the concentration calibration to be as large as a factor of two. A more precise calibration could be obtained using a confocal microscope.

RESULTS

NaCl Always Reversibly Decondenses and Softens Mitotic Chromosomes

Isolated chromosomes were extended to an initial force of $0.4\ \text{nN}$ and then sprayed with $30\text{--}500\ \text{mM}$ of NaCl for $10\ \text{s}$. A time series of images was acquired at $10\ \text{frames/s}$ for $40\ \text{s}$, where the spray was initiated about $5\ \text{s}$ into the time series (Fig. 2a,b). Following the initiation of the $10\ \text{s}$ spray, the force reduction equilibrated in about $2\ \text{s}$. The final change in force was determined by averaging the last $5\ \text{s}$ of the spray. Increasing the distance between the chromosome and the spray pipette reduced the concentration of ions. This was calibrated following the experiment as described in Materials and Methods. Spray experiments with 15 different concentrations were done.

NaCl always induced a decrease in the measured force (Fig. 3). Concentrations below $500\ \text{mM}$ of NaCl slightly decreased the force and density of the chromosome (Fig. 3). The maximum force and density occurs around $100\ \text{mM}$ of NaCl. After the chromosome was returned to physiological salt concentrations, the force constant was measured. Below $600\ \text{mM}$ NaCl,

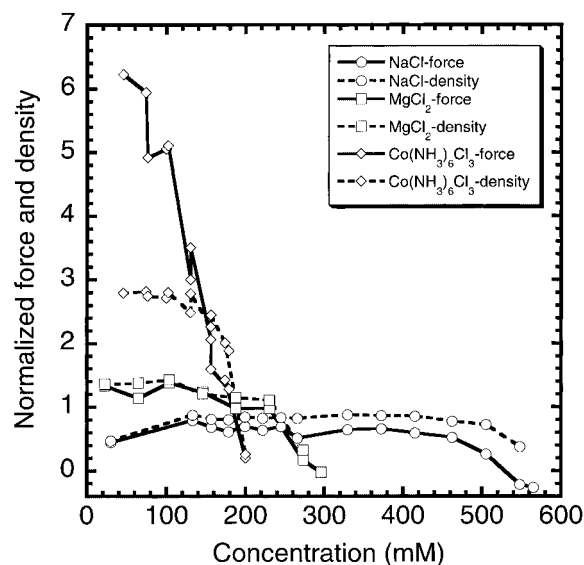


Fig. 3. The normalized force (solid line) and density (dashed line) of mitotic chromosomes vs. concentration of NaCl (\circ), MgCl_2 (\square), and $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (\diamond). The normalized force and width measurements are the steady-state force or density during a salt exposure divided by the initial force or density before the exposure. Since the force and density are normalized, the value of 1 implies no change is induced. NaCl always causes a decrease in the force and density with a minimum change at about $150\ \text{mM}$. However, MgCl_2 and $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ cause an increase in the force and density for concentrations below $100\ \text{mM}$ and a decrease in force and density above $150\ \text{mM}$. These results are qualitatively similar to the behavior of other charged biopolymers, dsDNA [Gosule and Schellmann, 1976] and actin [Tang and Janmey, 1996], and indicate that the chromatin inside mitotic chromosomes behaves as a charged biopolymer.

the force constant of the chromosome returns to a larger (stiffer) value (Table I).

We also exposed mitotic chromosomes to $2\ \text{M}$ NaCl and observe the same decondensation. For short exposures of $10\text{--}40\ \text{s}$, the chromosome fully recondenses with an increase in the force constant (Table I). However, following exposures of $> 250\ \text{s}$, the chromosome does not fully recondense and is irreversibly changed. The length is more than doubled, the force constant is reduced by more than half (Table I), and the contrast in the phase microscope is reduced. This reduction in force constant is especially dramatic because the length has more than doubled as well (the force constant is defined as the force required to double the chromosome length). The chromosome has a qualitatively different (far softer than native) mechanical response after long exposure to $2\ \text{M}$ NaCl. We emphasize that this irreversible behavior does not occur for the short exposures ($10\ \text{s}$) of lower

TABLE I. Chromosome Force Constants Before and After Microspraying

Ion	Maximum concentration (mM)	Exposure time (s)	Initial force constant (nN)	Final force constant (nN)	Change in force constant (nN)	% Change in force constant
Na ⁺	30	60	0.43	0.54	+0.11	+26
Na ⁺	300	20	0.32	0.49	+0.17	+53
Na ⁺	600	20	0.37	0.67	+0.30	+80
Na ⁺	2,000	30	0.55	0.65	+0.10	+27
Na ⁺	2,000	270	0.55	0.26	-0.29	-53
Mg ²⁺	20	60	0.75	1.39	+0.64	+85
Mg ²⁺	100	60	0.42	0.38	-0.04	-10
Mg ²⁺	300	20	0.67	0.72	+0.05	+07
Ca ²⁺	10	60	0.94	1.14	+0.20	+21
Co(NH ₃) ₆ ³⁺	200	40	0.54	0.38	-0.16	-30

ionic strength, which are the main focus of this study.

Above 250 mM Concentrations, MgCl₂ Reversibly Decondenses and Softens Mitotic Chromosomes, While Below 100 mM Reversibly Hypercondenses and Stiffens Mitotic Chromosomes

Chromosomes were extended so that they supported a force of about 0.5 nN, and were then exposed to between 10 and 300 mM of MgCl₂. Again, the force was monitored at a rate of 10 frames/s for 40 s, where the spray was initiated 5 s into the time series (Fig. 2c,d). Below 100 mM MgCl₂, the force and density increased by about 20% (Fig. 3). Between 100 and 200 mM MgCl₂, there is an initial increase in force (data not shown), followed by a return to the initial force (Fig. 3). At concentrations above 200 mM MgCl₂, the force drops nearly to zero, indicating complete loss of the ability for the chromosome to support force (Fig. 3). CaCl₂ (10 mM) also caused an increase in force identical to 10 mM MgCl₂. Larger concentrations were not studied because at higher CaCl₂ concentrations, calcium phosphate forms at the tip of the spray pipette.

Following a return to physiological salt conditions, the force constant was measured. Over this range of MgCl₂ concentrations, the force constant did not change by more than a factor of 2, indicating approximately reversible unfolding–refolding behavior.

Hexaminecobalt Trichloride Hypercondenses and Decondenses Mitotic Chromosomes

Using hexaminecobalt trichloride allows us to study how the cation valence affects chromosome condensation. Mitotic chromosomes were stretched so that they initially supported about 0.2 nN of force, and then exposed to concentra-

tions between 40 and 200 mM. Below 150 mM Co(NH₃)₆Cl₃, the force increases about five times, from 0.2 to 1 nN (Fig. 2e). This is qualitatively similar to MgCl₂, however, the effect is quantitatively much larger. Between 150 and 200 mM, this increase in force is reduced, and at 200 mM the force drops to near zero indicating the chromosome has lost the ability to support force (Fig. 3). Following the exposure to 200 mM Co(NH₃)₆Cl₃, the force constant is reduced by about 30%, indicating the chromosome did not fully recondense.

Force Supported by a Chromosome Is Related to Its Density

For each time series, the width, length, and a force was measured. By assuming the chromosome width is similar in both the horizontal and vertical directions, we calculated the volume of the chromosome, which is inversely proportional to the average chromosome density. Figure 3 plots how the force and density is related to the concentration of various ions. The density increases or decreases when the force increases or decreases, respectively. This implies that the denser the chromosome is, the more force it is able to support. However, density and force are not proportional, as can be seen for hexaminecobalt trichloride. The force continues to increase, while the density is remaining constant for decreasing concentration. This plateau in the density is likely due to the chromosome reaching its maximum degree of compaction.

Unconstrained Chromosomes Display Isotropic Expansion and Contraction

We independently measured the chromosome width and length chromosomes held in one pipette while the other end was free. A chromosome was isolated as described in Materials and

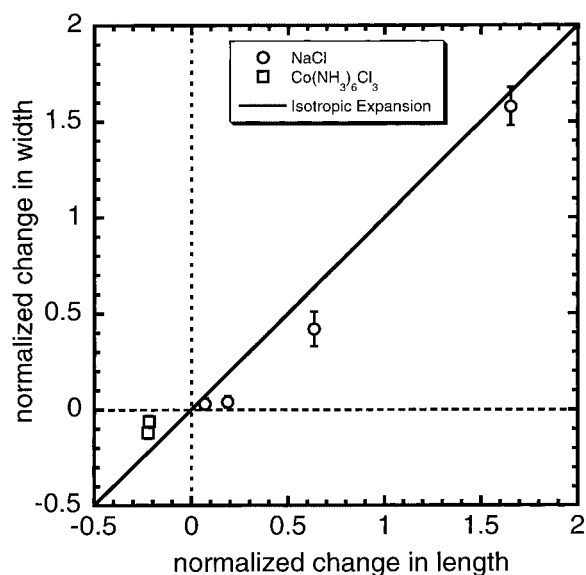


Fig. 4. Salt-driven isotropic decondensation and hypercondensation of mitotic chromosomes. The NaCl (○) concentrations used were 150, 200, 300, and 400 mM, where increasing concentration corresponds to increased length and width. The hexaminecobalt trichloride (□) concentrations used were 5 and 40 mM, where increasing concentration corresponds to decreased length and width. NaCl decondenses and hexaminecobalt trichloride hypercondenses mitotic chromosomes similarly to what is expected for isotropic contraction and expansion (solid line), and is not consistent with what is expected for chromatin loops tethered to a protein scaffold. This response is consistent with a cross-linked polyelectrolyte network. Also, the maximum condensation by hexaminecobalt trichloride shows that mitotic chromosomes are at least 1/3 aqueous solution.

Methods, and then exposed to increasing concentrations of NaCl or hexaminecobalt trichloride. NaCl concentrations of 150, 200, 300, and 400 mM and hexaminecobalt trichloride concentrations of 5 and 40 mM were used. Figure 4 shows that the decondensation and hypercondensation of mitotic chromosomes is roughly isotropic.

Hexaminecobalt Trichloride Reduces Chromosome Volume by 1/3

Figure 3 shows that as the concentration of hexaminecobalt trichloride is decreased below 100 mM, the density approaches a plateau, while the force continues to increase. Again, this implies that the chromosome is reaching a maximum density. At these concentrations, Figure 4 shows that a hypercondensed, unconstrained chromosome has a normalized change in length of 0.8 and normalized change in width of 0.9. This implies a decrease in volume by 1/3.

Also, after the concentration of $\text{Co}(\text{NH}_3)_6^{3+}$ is returned to zero, chromosome returns to its original volume with a similar force constant (Table I). This suggests that the hypercondensation involves the expulsion of aqueous solution, the native mitotic chromosome is only 2/3 of DNA and protein, and that 1/3 is essentially water.

DISCUSSION

We have shown that chromosome structure can be rapidly and reversibly hypercondensed and decondensed by shifts in ionic conditions on the second time scale. Combining our results with previous salt studies of chromatin and chromosomes allow us to understand how changes in ionic conditions perturb chromosome structure. The simplest explanation is that the rapid shifts in ionic conditions mainly perturb the chromatin level of structure. This is consistent with the reversibility in both the force constant and size of the chromosome.

Conversion of Chromatin Within the Chromosome From 30 to 10 nm Fiber Explains the Effects of Low NaCl Concentrations

Thirty millimolar concentrations of NaCl cause a decrease in the force supported by a mitotic chromosome and a decrease in chromosome density (Fig. 3). This can be understood in terms of low concentrations of NaCl ions effects chromatin structure. It was previously shown [Van Holde, 1988] that chromatin structure is gradually converted from the 30 nm fiber to the 10 nm fiber as NaCl concentrations are reduced below 75 mM. The elements maintaining chromosome condensation are not permanently interfered with, since there is little permanent change in chromosome force constant or size. Therefore, low NaCl concentrations partially unfold chromatin fibers while the higher order chromosome structure is relatively undisturbed.

It should be noted that the NaCl curves of Figure 3 approach, but do not quite reach native force and density levels (force and density reach peaks of about 80% of native near 100 mM NaCl). This slight undershoot can be understood easily, since our Tris-based spray buffer does not include the ~ 2 mM slightly condensing divalent metal ions present in our cell culture medium; thus, our NaCl spray experiments do not quite reach the native compaction.

Chromosome Decondensation At ~ 500 mM NaCl Can be Interpreted as Unfolding the Chromatin Fibers Without Removing Histones

Mitotic chromosomes are unable to support tension and the density dramatically decreases above 500 mM NaCl. This is where core histones begin to be removed from nucleosomes [Van Holde, 1988]. However, histone removal must be done by gradually increasing the NaCl concentration over a period of at least an hour. A rapid increase of NaCl concentration to above 500 mM does not remove core histones from nucleosomes for at least 40 min [Yager et al., 1989]. However, experiments exposing chromatin fibers to 500 mM NaCl for short periods show a shift in the sedimentation coefficient, which is due to a destabilizing of the nucleosome without removal [Van Holde, 1988].

The decondensation of mitotic chromosomes observed for a NaCl concentration of 500 mM is, therefore, likely due to destabilization of chromatin fiber structure. Higher levels of mitotic chromosome structure are likely not permanently removed by this salt treatment, since the chromosome returns to near-native size and force constant following the removal of the salt treatment.

Chromosome Hypercondensation at Millimolar Concentrations of Multivalent Ions is Consistent With an Induced Attraction Between Chromatin Fibers

Low concentrations (~ 10 mM) of multivalent ions cause an increase in the force supported by a chromosome and a decrease in its width. It was previously shown that chromatin aggregates above 1 mM of $MgCl_2$ [Borochoy et al., 1984] and 0.01 mM hexaminecobalt trichloride [Sen and Crothers, 1986; Smirnov et al., 1988]. This suggests that the decrease in volume and increase in force are due to an induced attraction between chromatin fibers, which is mediated by the multivalent ions.

This attraction is consistent with aggregation of other biopolymers such as DNA [Gosule and Schellmann, 1976] and actin [Tang and Janney, 1996]. Theories which explain this aggregation have been described by Bloomfield [1991], Ray and Manning [1994], and Nguyen et al. [2000]. From this point of view, mitotic chromosomes are another biopolymer system for which multivalent ions induce like-charged polymers to attract each other.

Chromosome Decondensation at ~ 200 mM of Multivalent Ions is Explained by Destabilization of Chromatin Fibers

Mitotic chromosomes are unable to support an applied force and increase in width two times (Fig. 2d) for multivalent ion concentrations above 200 mM. The increase in width and reduction of force is similar to that obtained using 500 mM NaCl. There have been only a few studies of the effect of high concentrations of multivalent ions on chromatin fiber structure to compare our results with. Aaronson and Woo [1981] used turbidity to measure to compaction of chromatin within rat liver nuclei and found that between 2 and 30 mM of $MgCl_2$, the chromatin is maximally compacted. The chromatin is over compacted up to 100 mM, at which it appears to decondense as measured by turbidity. These results suggest that the decondensation at 200 mM is due to destabilization of the chromatin fibers, analogous to the decondensation at 500 mM NaCl.

Longer-Duration Exposures to 2 M NaCl Concentrations Lead to Irreversible Modification of Chromosome Structure and Elasticity

As described above, short-duration exposures to NaCl and $MgCl_2$ gave rise to reversible unfolding–refolding behavior, the main focus of this study. To qualitatively check that irreversible changes in chromosome structure could eventually occur as a result of screening out of electrostatic interactions, we carried out longer (4 min) exposures of high (2 M) NaCl. We observed irreversible unfolding and reduction of elastic modulus, which is easily explained in terms of known destabilization of histone–DNA interactions by long exposures to high NaCl concentrations [van Holde, 1988; Yager et al., 1989]. This explanation is of course tentative, since we did not assay histone concentration, which could be done using, e.g., labeled anti-histone [Poirier et al., 2000]. It would also be interesting to carry out irreversible-unfolding experiments using high concentrations of $MgCl_2$ and other ions, although NaCl is most relevant to the existing literature on destabilization of histone–DNA interactions.

In addition to these conclusions regarding the effect of ionic strength on mitotic chromosome structure, we have come to several more general conclusions.

Metaphase Chromosomes Are 1/3 Aqueous Solution

Using condensing agents, we have seen that 1/3 of the mitotic chromosome volume can be removed in a few seconds and then replaced in at most a few minutes. The only reasonable explanation for this is that at least 1/3 of the chromosome is actually water and other small, mobile molecules, which can be squeezed out when strong attractions between chromatin fibers are introduced. In turn, this indicates that native chromatin–chromatin interactions in mitotic chromosomes are not strongly attractive.

Mitotic Chromosomes Can be Rapidly and Reversibly Reorganized

The mitotic chromosome swelling and compaction that we have observed is remarkably rapid: mitotic chromosomes can be expanded six times their native volumes and condensed 2/3 of their native volumes in about a second. Chromosomes can then be returned to native conformations with a native elastic response simply by returning the solution ionic strength to native values.

This ability for mitotic chromosomes to be rapidly reorganized suggests that mitotic chromosome structure is not highly ordered in the sense that each region of chromatin does not have a regulated folded pattern. A highly ordered chromosome (i.e., with a precisely controlled three-dimensional structure) should require appreciable time to anneal away the disorder introduced by the gross unfolding transitions observed in this study.

Mitotic Chromosomes Are Folded in an Isotropic Fashion

Chromatin domains are free to expand outwards away from the chromosome axis and along the chromosome's length, indicating a globally isotropic organization of mitotic chromosomes. Our results are inconsistent with a highly anisotropic chromosome organization, most notably chromatin loops emanating from a contiguous protein 'scaffold'. It must be kept in mind that structural interpretation of the isotropic response shown in Figure 4 will require additional biochemical experiments to rule out, e.g., the possibility that the scaffold itself is reversibly and rapidly disassembled, and then reassembled by ionic condition shifts.

This study has focused on use of shifts in ionic conditions to provide information about the structure of mitotic chromosomes. However, we note that changes in electrostatic interactions of chromatin may also be physiologically relevant. There is evidence that a charge patch on histone H1 is controlled by phosphorylation and plays a role in controlling gene regulation [Dou and Gorovsky, 2000]. Also, a charge patch on the histone variant H2A.Z may be regulated by acetylation and appears to play an essential function in *Tetrahymena*, [Ren and Gorovsky, 2001].

In summary, electrostatic interactions clearly play a crucial role in maintaining native mitotic chromosome structure. Disruption of electrostatic interactions leads to rapid, isotropic swelling behavior similar to that of a crosslinked polyelectrolyte network. We find no evidence for a globally anisotropic structure of mitotic chromosomes such as remnant elasticity after chromatin is unfolded or anisotropic swelling behavior. Finally, we find that there is an appreciable amount of mobile aqueous solution inside mitotic chromosomes.

ACKNOWLEDGMENTS

We thank J. Widom for helpful discussions, and we are indebted to D. Reese for generously sending us the TVI cells used in this study.

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