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Mechanical Continuity and Reversible Chromosome Disassembly Within Intact Genomes Removed From Living Cells

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Abstract Chromatin is thought to be structurally discontinuous because it is packaged into morphologically distinct chromosomes that appear physically isolated from one another in metaphase preparations used for cytogenetic studies. However, analysis of chromosome positioning and movement suggest that different chromosomes often behave as if they were physically connected in interphase as well as mitosis. To address this paradox directly, we used a microsurgical technique to physically remove nucleoplasm or chromosomes from living cells under isotonic conditions. Using this approach, we found that pulling a single nucleolus or chromosome out from interphase or mitotic cells resulted in sequential removal of the remaining nucleoli and chromosomes, interconnected by a continuous elastic thread. Enzymatic treatments of interphase nucleoplasm and chromosome chains held under tension revealed that mechanical continuity within the chromatin was mediated by elements sensitive to DNase or micrococcal nuclease, but not RNases, formamide at high temperature, or proteases. In contrast, mechanical coupling between mitotic chromosomes and the surrounding cytoplasm appeared to be mediated by gelsolin-sensitive microfilaments. Furthermore, when ion concentrations were raised and lowered, both the chromosomes and the interconnecting strands underwent multiple rounds of decondensation and recondensation. As a result of these dynamic structural alterations, the mitotic chains also became sensitive to disruption by restriction enzymes. Ion-induced chromosome decondensation could be blocked by treatment with DNA binding dyes, agents that reduce protein disulfide linkages within nuclear matrix, or an antibody directed against histones. Fully decondensed chromatin strands also could be induced to recondense into chromosomes with pre-existing size, shape, number, and position by adding anti-histone antibodies. Conversely, removal of histones by proteolysis or heparin treatment produced chromosome decondensation which could be reversed by addition of histone H1, but not histones H2b or H3. These data suggest that DNA, its associated protein scaffolds, and surrounding cytoskeletal networks function as a structurally-unified system. Mechanical coupling within the nucleoplasm may coordinate dynamic alterations in chromatin structure, guide chromosome movement, and ensure fidelity of mitosis. J. Cell. Biochem. 65:114–130.

Key words: chromatin; histone; mitosis; nuclear matrix; nucleolus; micromanipulation; tensegrity

Chromosomes are commonly viewed as mechanically separate units, largely based on their discrete appearance as seen in conventional metaphase "squash" preparations. This perception of chromosomes as physically separate entities is supported by the finding that meiotic chromosomes can be isolated individually from insect cells using a microneedle and that they behave as individual units when detached from the spindle and manipulated in situ [Nicklas, 1967; Nicklas and Kubai, 1985]. Biophysical analysis of DNA molecules isolated from mitotic Drosophila cells also suggests that the length of the longest molecules in solution corresponds to the calculated size of the largest metaphase, anaphase, or G1 chromosome [Kavenoff and Zimm, 1973]. This view of chromosomes as isolated structures is supported by the finding that chromosomes are randomly positioned within the metaphase rosettes of male muntjak cells [Korf and Diacumakos, 1977].

However, other findings conflict with this idea that chromosomes are isolated structures. Many investigators have visualized filamentous con-

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nections between different chromosomes at both the light and electron microscopic levels [DuPraw, 1965; Hoskins, 1965, 1968; Diacumakos et al., 1971; Takayama, 1975; Burdick, 1976; Garcia-Blanco et al., 1995]. These mitotic threads label with ^{3}H-thymidine, suggesting that they actually may be composed of DNA [Myhra and Brogger, 1975]. Ultraviolet microbeam irradiation studies of meiotic cranefly spermatocyte chromosomes demonstrate that autosomes, sex chromosomes, and partner halfbivalents functionally influence each other's movements via a mechanism that is independent of the mitotic spindle [Forer, 1966; Yin and Forer, 1996]. Lengths of DNA in polytene chromosomes, giant mitotic insect neuroblasts, and in certain other Drosphila cells during S phase have been found to be longer than that calculated for the largest chromosomes in these cells [Kavenoff and Zimm, 1973]. Furthermore, in contrast to studies which show randomly positioned chromosomes in muntjak cells [Korf and Diacumakos, 1977], chromosomes in many different types of human cells appear to be positioned in regular locations relative to one another and to be reintegrated in precise locations after each mitotic period [Manuelidis, 1990; Miller et al., 1963a,b; Nagele et al., 1995].

Structural continuity within the cytoplasm and nucleus also has been revealed by extracting cells with chemical buffers containing detergents, nucleases, and high salts to uncover the insoluble nuclear matrix-cytoskeletal scaffold [Fey et al., 1984; Nickerson et al., 1992]. The nuclear matrix is an anastomosing continuum of complex molecular composition that is comprised of "core filaments" that are enriched in ribonucleoprotein, resistant to DNase treatment, and highly cross-linked by disulfide bonds [Berezney and Coffey, 1975; Capco et al., 1982; Mirkovitch et al., 1984; Jackson and Cook, 1985; Pienta et al., 1991; Nickerson et al., 1992]. In electron microscopic studies of interphase cells, the nuclear matrix appears to physically interconnect different nuclear components (e.g., nucleoli) to each other and to the surrounding cytoskeleton [Fey et al., 1984]. During mitosis, the nuclear matrix interconnects chromosomes and centrosomes [Nickerson et al., 1992], and appears to pattern proteins associated with the mitotic apparatus (e.g., NuMA, Cemp-B, Cemp-F, Rb) [Mancini, 1996]. Additional structural control could be provided by other nuclear proteins, including histones, which play a key role in maintaining chromatin form [Thoma et al., 1979; Woodcock et al., 1984; Bickmore and Oghene, 1996].

Finally, recent studies show that chromosomes and nucleoli change their location and exhibit molecular realignment in living cells when cell surface integrin receptors and interconnected cytoskeletal filaments are mechanically stressed [Maniotis et al., 1997]. These results, combined with past studies using membrane-permeabilized cells [Sims et al., 1992], strongly support the possibility that different intranuclear components are mechanically coupled to each other and to the surrounding cytoskeleton in situ. Thus, the question of how the genome is organized and integrated within the cell remains unanswered.

In this study, we describe a microsurgical method for removing the intact genome from living mammalian cells under physiological conditions. Using this approach in combination with different enzyme treatments and chemical extraction procedures, we demonstrate that the entire genome is physically connected and mechanically coupled to the surrounding cytoskeleton, even in mitotic cells. Furthermore, condensed chromatin microsurgically removed from living interphase or mitotic cells retains the ability to undergo dynamic structural transformations in response to changes in ionic concentration or modification of DNA/histone interactions, such that entire chromosomes can be seen to disassemble and reassemble in real-time. Analysis of the molecular basis of chromosome continuity and dynamic plasticity reveal that DNA is responsible for mechanical coupling throughout the genome and histone H1 drives chromosome assembly whereas ionic concentrations and disulfide-linked nuclear proteins dictate higher order chromosome pattern.

MATERIALS AND METHODS

Experimental System

Bovine or human capillary endothelial cells were cultured on gelatin-coated glass coverslips in complete medium containing DMEM (Sigma, St. Louis, MO), 10% calf serum, 25 mM Hepes (pH 7.4; JRH Biosciences), and endothelial mitogen [Ingber, 1990]. Cells were placed within 35 mm plastic dish lids containing 2 ml of DMEM and allowed to equilibrate at 37°C in a 10% CO_2 buffered incubator before micromanipulation. For optimal microsurgical conditions, cells were grown to near confluence, which

produced strong attachments needed to resist the pulling forces associated with rapid (within 1 sec) micropuncture and removal of nuclear components without causing cell detachment. In contrast to past chromosome micromanipulation studies that were carried out at high pH (8.0) [Korf and Diacumakos, 1977, 1980], all experiments were carried out under isotonic culture conditions in DMEM at pH 7.4; similar results also were obtained using complete medium.

Nucleoli and chromosomes were "harpooned" within living cells by rapidly piercing the nucleus directly over a nucleolus or on the side of a mitotic plate with a glass microneedle and then laterally drawing out the contents through the hole created by the micropuncture using a Leitz micromanipulator. In certain studies which utilized treatments that cause cell detachment (e.g., SDS, proteases), the entire chromosome chains were removed from the cell and attached to a free area of the culture substrate while still adherent to the pipette. The dishes were held in an Omega RTD 0.1 stage heating ring coupled to a Nikon Diaphot inverted microscope fitted with a 60× long working distance objective and overlayed with thin layer of mineral oil. Narishige micropipettes were pulled with a Sutter micropipette puller adjusted to produce long tips approximately 1–5 um wide along a length of 40–100 um (tip widths were consistently less than 0.5 um).

Microscopy and Data Analysis

Microscopic images were captured with a DAGE MTI camera and recorded in real time using a Gyrr videotape recorder in conjunction with a Macintosh Quadra 800 computer and Oncor Image Analysis software. Changes in chromosome morphology were quantitated using an integration program (BDS Image software) by superimposing density displays of the chromosome or chromosome chains before and after exposure to a particular reagent. Differences in pixel density were then translated into an integrated picture of the phase density overlap for quantitation. Images were converted to PICT format for presentation using ADOBE Photoshop software.

Reagents and Digestions

Each compound to be tested was added in a 1 ul droplet directly above the extended strand (i.e., to a total of 2 ml DMEM) and changes in chromosome morphology were quantitated through the videomicroscope in real-time. The reagents tested included: DNase I (1–20 units/droplet), Eco R1 (5 to 50 units), Hind III (5 to 10 units), Hind III (5 to 10 units), (Boehringer-Mannheim); micrococcal nuclease (5 units-Sigma), Bam H1 (5 to 10 units-New England Biolabs), RNase A (1–100 units-Sigma), RNase 1 (1–100 units-Promega), proteinase K (50 ng to 20 ug-Boehringer-Mannheim), trypsin (5 ug-Gibco), and heparin (10 ug-Sigma). Final concentrations of other compounds tested were: YoYo (150 nM-Molecular Probes), ethidium bromide (1 to 5 ug/ml), Hoechst 33258 (0.5 to 10 ug/ml), sodium dodecyl sulfate (SDS; 3%), ammonium sulfate (0.25 M), dithiothreitol (5 to 100 mM), β-mercaptoethanol (1 mM), triton-X-100 (0.5%), nocodazole (1 to 10 ug/ml), colchi-

Fig. 1. Phase contrast (**A–P**) and fluorescence (**Q**) micrographs demonstrating mechanical continuity with the nucleoplasm of living interphase (A–E) and mitotic (F–Q) endothelial cells. A–E: An interphase cell in which a single nucleolus was harpooned with a glass micropipette (arrow) and progressively drawn out of its nucleus to the right over a period of 3 sec. Pulling on a single nucleolus resulted in subsequent removal and elongation of all of the nucleoli which appeared to be part of one continuous thread. F–J: A mitotic cell in which a single metaphase chromosome was harpooned and progressively pulled out to the right. Arrow in (G) indicates the tip of the pipette attached to a single chromosome at the beginning of the pull; further pulling resulted in sequential removal of all of the chromosomes as part of an interconnected chain (H). I: When viewed at lower magnification, all the microsurgically removed chromosomes shown in (H) appeared to be interconnected by a continuous string (arrows) which was most visible when the strand was held under tension. J: When the tension was released in the extended strand shown in (I) by moving the pipette to the left, shortening of the interchromosomal spaces and elastic retraction of the entire mitotic chain resulted, but chromosomes did not appear to change their relative spacing or individuality when tension was re-applied. K–M: Harpooning a single chromosome in one chromosome plate of an anaphase cell (shown at top left) resulted in progressive removal of both plates, demonstrating maintenance of mechanical coupling between different chromosome plates even when initially separated in the living anaphase spindle. N–P: When a single chromosome was harpooned within a cell in telophase, one chromosome plate (arrow) was first extended as a thread and then fully removed from the cell without altering the orientation or position of the second plate, demonstrating loss of continuity between the two daughter genomes at this point in the cell cycle. Q: Chromosome chain removed from endothelial cell during colchicine-induced mitosis and then labeled with Hoechst 33258 dye (0.5 ug/ml for 5 min) demonstrating that both the condensed chromosomes and the interchromosomal strands stained positively for DNA (A–H, K–M; ×1000; I, J: ×400; N–P: ×1,400; Q: ×2,200).

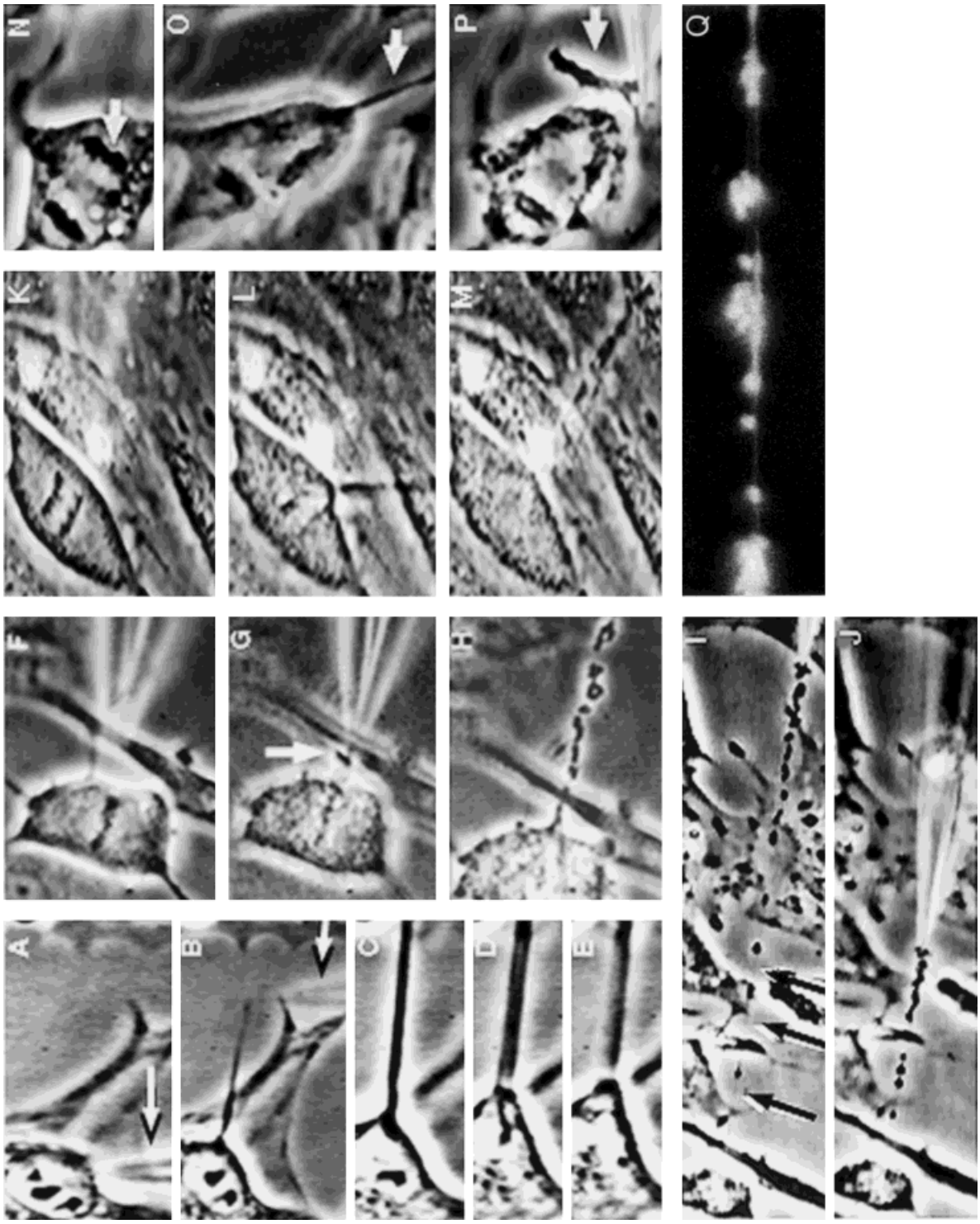

Figure 1.

cine (1 to 10 ug/ml), taxol (1 uM), cytochalasin D (1 to 5 ug/ml), phalloidin (200 nM), acrylamide (5 mM) (Sigma), and gelsolin (2 mg/ml; kindly provided by Dr. Paul Janmey). All reagents were adjusted to neutral pH, and all diluents and buffers were tested and shown to have no significant effect on chromatin decondensation or condensation.

RESULTS

Physical Removal of Nucleoplasm and Chromosomes

To directly test whether discrete networks physically interlink chromatin, we used very fine glass microneedles (tips less 0.5 μm diameter) to harpoon individual nucleoli within cultured endothelial cells. After harpooning a nucleolus, we almost instantaneously (<1 sec) pulled it out from the nucleus and cell through the micropuncture site we created. Pulling this single nucleolus resulted in coordinated movements of other more distant nucleoli. This culminated in progressive removal of all of the nucleoli which appeared as large masses interconnected by single continuous nucleoplasmic thread (Fig. 1A–E).

As a control, we then attempted to demonstrate discontinuity within chromatin by harpooning mitotic chromosomes. However, all the chromosomes always were removed sequentially as if they were beads on an elastic thread, during prophase (this result was observed in 100 out of 100 consecutive pulling experiments), metaphase (Fig. 1F–J; n = 805/805), and anaphase (Fig. 1K–M; n = 60/60). As a chromosome was pulled from a cell, neighboring chromosomes always appeared to be physically coupled, both inside and outside the cell. Also, the two chromosome plates behaved as if they were a single interconnected structure in early anaphase. In contrast, a single chromosome plate could be selectively removed using the harpooning procedure during telophase (Fig. 1N–P; n = 41/41), indicating that chromosome plates disconnect at this time and confirming that the nuclear continuity we observed was not due to the nuclear harpooning procedure. Furthermore, telophase cells that had one of their plates microsurgically removed could divide into two daughter cells (one with a nucleus, the other without; not shown), confirming that the microsurgery does not irreversibly injure the mitotic spindle or kill the cell, as previously described [Li and Nicklas, 1995].

In the past, it has been suggested that the connections between different chromosomes observed after chromosome removal may be due to non-specific stickiness [Korf and Diacumakos, 1980]. These older studies employed an oil-based extraction technique or microsurgery at high pH to remove chromosomes [Diakumacos et al., 1971; Korf and Diacumakos, 1978]. In the present study, we were able to circumvent potential artifacts, such as chromosome collisions, using a rapid harpooning method under physiological culture conditions. Under these conditions, the physical connections between different chromosomes clearly were not due to non-specific adhesions. First, chromosome morphology and number did not change after surgical removal in isotonic medium. Second, spatially isolated chromosomes still visible within the cells's mitotic plate inside the cell remained stationary until the tension of the thread pulled them out. Third, when the extended mitotic chain was allowed to elastically retract and collapse into a single clump of chromatin and then tension was reapplied via the attached micropipette, all of the clumped chromosomes separated and once again became morphologically distinct and returned to their original separated positions (Fig. 1F–M). Fourth, two or more freshly isolated mitotic chains did not stick to each other when brought into direct contact in the culture medium even though non-specific chromosome aggregation could be induced under the same culture conditions by intentionally damaging (crushing) the chromosomes using the micropipette. Finally, and most significantly, high temperature (60°C for 2–4 h or 100°C for 10 min) [Kavenoff and Zimm, 1973] in the presence of 50–80% formamide did not disrupt chains under tension (n = 10/10), although partial loss of chromosome morphology was observed.

Characterization of Interchromosomal and Nucleoplasmic Continuity

Past studies have revealed the presence of interchromosomal fibers [Hoskins, 1965, 1968; Diacumakos et al., 1971], and have shown that living cells incorporate tritiated thymidine into these connections [Myhra and Brogger, 1975]. Indeed, in experiments in which we labeled chromosome chains during microsurgical removal with DNA-binding dyes (e.g., Hoechst, ethidium bromide, DAPI), we noted that two thin threads were observable at the limits of

resolution between larger chromatin densities, even in endothelial cells pre-incubated in colchicine or nocodazole (Fig. 1Q). To explore the molecular basis of these connections, extended nucleoplasmic threads were exposed to different enzymes, chemicals, and extraction conditions (Table I). Treatment with DNase I and micrococcal nuclease caused interphase threads to abruptly lose their continuity and retract (Fig. 2A,B). When mitotic threads were analyzed, the same nucleases produced progressive thinning, elongation, and eventual breakage (Fig. 2C–F). These breaks preferentially occurred in regions of the thread that stretched between adjacent chromosomes (Fig. 2C–E). In contrast, when native interphase and mitotic threads were exposed to RNases (types A, 1) or extracted with 0.5% Triton-X-100, strand conti-

TABLE I. Mechanical Continuity and Structural Memory in Mitotic Strands*

Response Treatment	Disrupts native strand	Decondenses with high Mg^{+2}	Recondenses	Disrupts open strand	Destroys pattern
DNA Modulators—					
DNase I	+ (17/17)	N.D.	N.D.	N.D.	+
Micrococcal nuclease	+ (20/20)	N.D.	N.D.	N.D.	+
Eco R1	− (7/7)	+	+ (one time only)	+	+
Bam	− (1/1)	+	+ (one time only)	+	+
Hind III	− (3/3)	+	+ (one time only)	+	+
Ethidium bromide	− (9/9)	−	N.D.	N.D.	+
Hoechst 33258	− (10/10)	−	N.D.	N.D.	+
YoYo	− (3/5)	−	N.D.	N.D.	+
Formamide + heat	− (18/18)	+ (without Mg^{+2})	N.D.	N.D.	+
RNA Modulators—					
RNase A	− (8/8)	N.D.	N.D.	N.D.	N.D.
RNase 1	− (4/4)	+	+	−	−
Protein Modulators—					
SDS	− (6/6)	+ (without Mg^{+2})	−	−	+
DTT or β-MCE	− (9/9)	+	−	−	+
Proteinase K (low)	− (12/12)	+ (without Mg^{+2})	− (+ with H1)	−	+ (− with H1)
Trypsin	− (26/26)	+ (without Mg^{+2})	− (+ with H1)	−	+ (− with H1)
Heparin	− (14/14)	+ (without Mg^{+2})	− (+ with H1)	−	+ (− with H1)
Proteinase K (high)	+ (5/5)	N.D.	N.D.	N.D.	+
Lipid Modulator—					
Triton-X-100	− (4/4)	+	+	−	−
Cytoskeletal Modulators—					
Nocodazole	− (3/3)	+	+	−	−
Colchicine	− (3/3)	+	+	−	−
Taxol	− (10/10)	+	+	−	−
Cytochalasin D	− (4/4)	+	+	−	−
Phalloidin	− (3/3)	+	+	−	−
Acrylamide	− (3/3)	+	+	−	−
Gelsolin	+ (6/8)	N.D.	N.D.	N.D.	+

*+ Indicates the treatment produced the response described at the top of each vertical column; − indicates no response. Disrupts Native Strand, causes the microsurgically removed strand to break without decondensation; Decondenses with High Mg^{+2}, does not interfere with the decondensing effects of magnesium; Recondenses, reforms pre-existing strand morphology as the magnesium dilutes in the surrounding medium; Disrupts Open Strand, causes strand breakage only after decondensation is induced; Destroys Pattern, produces loss of chromosome form and genomic arrangements; without Mg^{+2}, indicated response did not require the presence of magnesium; + with H1, produces this effect only when histone H1 is present; N.D., not done. Numbers in parentheses indicate the number of experiments that produced these responses/total number experiments. Compounds added directly above the mitotic strand in a 1 ul droplet include: DNase I (1–20 units), micrococcal nuclease (5 units), Eco R1 (5 to 50 units), Bam (5 to 10 units), Hind III (5 to 10 units), RNase A (1–100 units), RNase 1 (1–100 units), proteinase K (LOW = 50 ng/HIGH = 20 ug), trypsin (5 ug), and heparin (10 ug). Final concentrations of other compounds tested are: ethidium bromide (1 to 5 ug/ml in final DMEM), Hoechst 33258 (0.5 to 10 ug/ml), YoYo (150 nM), SDS (3%), formamide and heat (60% at 60°C for 4 h or 80% at 100°C for 10 min), dithiothreitol (DTT; 5 to 100 mM), β-mercaptoethanol (β-MCE; 1 mM), Triton-X-100 (0.5%), nocodazole (1 to 10 ug/ml), colchicine (1 to 10 ug/ml), taxol (1 uM), cytochalasin D (1 to 5 ug/ml), phalloidin (200 nM), acrylamide (5 mM), and gelsolin (2 mg/ml).

nuity and pattern integrity were always maintained (Table I). On the other hand, solubilization of nuclear proteins with 3% SDS, 0.25 M amonium sulfate, or high concentrations of proteinase K (20 mg/ml) resulted in complete loss of chromosome form (Fig. 2G–I), yet the disorganized material remained mechanically continuous, and capable of transferring force exerted by the attached microneedle (Fig. 2H,I).

Selective cytoskeletal-modulating drugs were used to test whether microtubules, microfilaments, or intermediate filaments are required for maintenance of continuity within mitotic threads. No effect on strand form or continuity could be demonstrated when treated with either nocodazole, colchicine, taxol, cytochalasin D, phalloidin, or acrylamide (Table I), confirming that neither spindle microtubules nor other cytoskeletal filaments are required for mechanical coupling between different chromosomes in these cells. In contrast, addition of the microfilament-severing protein, gelsolin, produced rapid and acute disruption of mitotic strands, specifically at their proximal end where the strands attach to the surrounding cytoplasm (Fig. 2J,K; Table I). However, continuity between different chromosomes was maintained in the presence of gelsolin, as indicated by our ability to successfully reextend the collapsed chain by applying tension to the micropipette. This disruption appeared to be due to breakage of cytoplasmic (i.e., rather than nucleoplasmic) connections since gelsolin had no effect on nucleoplasmic strands from interphase cells (n = 5/5) which retain an intact nuclear envelope that physically separates the nucleoplasm from the cytoplasm.

Ionic Sensitivity of Chromatin In Situ

In the course of these studies, we observed that entire chromosome chains and nucleoplasmic strands removed from living cells retained the ability to reversibly decondense and recondense in a coordinated manner in response to changing ion concentrations. In other words, the entire interconnected genome appeared to exhibit a "structural memory," such that preexisting chromatin pattern was always preserved. For example, adding one microliter of concentrated magnesium solution (500 mM $MgCl_2$) directly above an extended chromosome chain resulted in simultaneous decondensation of all of the chromosomes, followed by recondensation within 1 to 2 min as the cation diluted in the surrounding culture medium (n = 59/59). This was visualized by the progressive loss and then reappearance of the entire phase dense mitotic strand (Fig. 3A–C). Impressively, both the distinct forms of individual chromosomes and higher order genomic pattern were consistently restored, such that almost every chromosome reappeared in its previous location and maintained its characteristic spatial distribution relative to its neighbors. Furthermore, multiple oscillatory decondensation-recondensation cycles could be produced by repeatedly adding drops of magnesium (Fig. 3A–G) or other divalent and monovalent cations (e.g., 1 ul of 500 mM $CaCl_2$; n = 3/3; 1 M $CuCl_2$; n = 1/1; 1 M NaCl, n = 5/5; 1 M LiCl, n = 2/2) to native strands or strands extracted with Triton-X-100. Indeed, more that 20 repetitive cycles have been obtained with a single thread without compromising strand continuity or producing a major loss of pattern integrity. Decondensation also was induced by raising the final cation concentration (30 to 100 mM $MgCl_2$; n = 20/20) and this could be reversed by washing. Use of much higher final cation concentrations resulted in irreversible loss of both strand pattern and memory (1 M $MgCl_2$, n = 6/6; 2 M NaCl, n = 6/6). Furthermore, this memory was not limited to the mitotic chromosomes; similar reversible decondensation was observed in nucleoplasmic strands from interphase cells when magnesium was added (Fig. 3H–J; n = 17/17).

Fig. 2. Phase contrast micrographs demonstrating disruption of mechanical continuity in nucleoplasmic strands microsurgically removed from living interphase (**A,B**) and mitotic (**C–K**) cells. A,B: Treatment of interphase strands with DNase I (10 units/1 ul drop added above the strand) resulted in disruption and retraction of the broken ends (small arrows) of the strand. C–F: The same DNase I treatment produced a progressive increase in the length of the threads linking neighboring chromosomes and eventual breakage in these regions. Arrows indicate two neighboring chromosomes that progressively separate from one another before the strand breaks (E) and fully retracts (F). G–I: Extraction of chromosome chains (G) with 3% SDS resulted in loss of chromosome form and strand morphology (H). However, mechanical continuity was retained, as indicated by narrowing and elongation of the nucleoplasmic halo in response to downward displacement of the pipette tip (I). J,K: Treatment of mitotic strands with gelsolin (2 mg/ml) for 10 sec resulted in disconnection of the mitotic strand from its point of interconnection with the cell body. This break caused the entire elastic chromosome chain to retract back toward the pipette tip below which is not visible in this view. Arrow indicates where breakage occurs at the top of the chromosome chain before (J) and after (K) gelsolin-induced retraction. (A,B,J,K: ×700; C–F: ×1000; G–I: ×1300.)

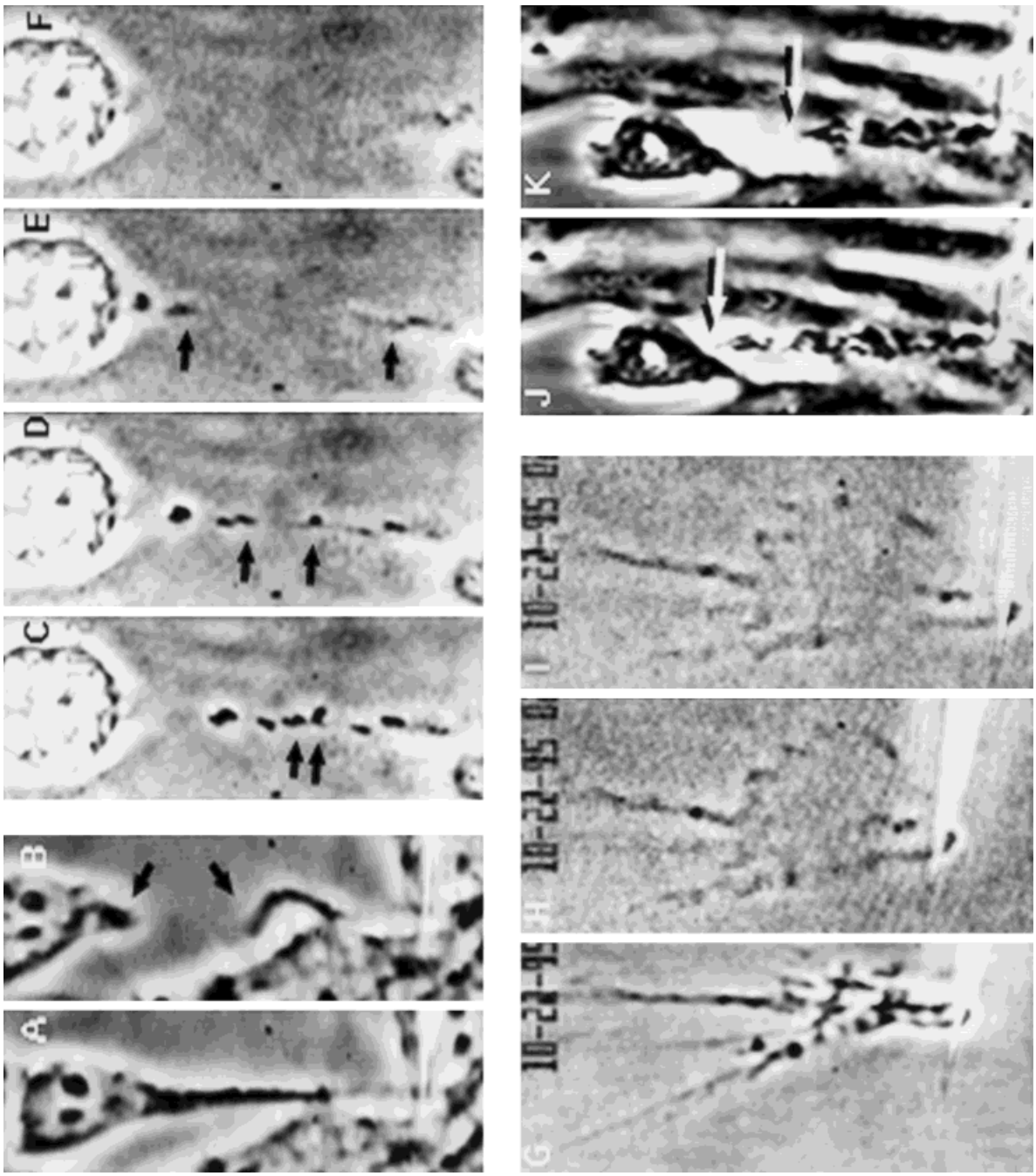

Figure 2.

Because we could reversibly decondense and condense chromosomes by manipulating ionic concentration, we exposed the chains to restriction endonucleases that nick double stranded DNA at specific sites and tested if these enzymes could disrupt chromatin continuity or alter pattern integrity under these conditions. Mitotic strands that were treated EcoR1, Bam H1, or Hind III initially remained intact, and could withstand one single round of decondensation and recondensation by adding magnesium. However, breakage of mitotic strands always resulted when a second round of decondensation was induced (Table I).

Nuclear matrix scaffolds which appear to physically interconnect chromosomes at the ultrastructural level [Nickerson et al., 1992] are stabilized by multiple protein disulfide linkages [Berezney and Coffey, 1975]. We therefore used disulfide-reducing agents (dithiothreitol, β-mercaptoethanol) to explore whether these nuclear protein scaffolds are required for maintenance of the mechanical continuity or structural memory we observed. Disruption of disulfides did not produce strand breakage. However, when strands treated for short times (5 to 10 mM DTT for 15 to 20 min) were decondensed by subsequent addition of magnesium, they failed to recondense normally as the cation diluted into the surrounding medium, and thus loss of normal genomic pattern resulted (Fig. 3K,L). Furthermore, longer exposures (30 to 40 min) to disulfide reducing agents produced condensation and clumping of chromosomes within native strands as well as complete and irreversible loss of chromosome form in response to magnesium addition, yet mechanical continuity was retained (Table I). Treatment of condensed strands with high salts or 0.25 M ammonium sulfate, which are major extraction steps in many nuclear matrix extraction procedures [Mirkovitch et al., 1984; Capco et al., 1982; Jackson and Cook, 1985; Nickerson et al., 1992] resulted in complete and immediate loss of chromosome form (Table I).

Pattern integrity and ionic sensitivity also depended on DNA flexibility. Treatment of decondensed strands with low concentrations of DNA-binding dyes (e.g., ethidium bromide, Hoechst 33258, and to a lesser degree, Yo-Yo), prevented these structural interconversions (Table I). Dye binding altered strand organization and greatly reduced its elasticity during or after chain removal, as indicated by loss of chromosome morphology during removal in the presence of the dye, and failure of the strand to retract elastically when tension on the pipette was released (Fig. 3M–O).

Histone H1-Dependent Chromosome Recondensation

While histone H1 has been shown to play a critical role in chromatin organization in studies with isolated DNA [Thoma et al., 1979], its role in chromosome condensation remains controversial [Ohsumi et al., 1993]. Thus, we used our novel model of in situ analysis to explore the role of histones in chromosome condensation within native chromatin. Microsurgically-removed mitotic chains were decondensed by adjusting the final concentration of $MgCl_2$ in the medium to 50 to 300 mM. Incubation of these decondensed strands with monoclonal antibodies against histones resulted in almost complete restoration of chromosome shape, size, number, and position along the reformed mitotic chain (greater than 95% recovery as determined by computerized image analysis; Fig. 4A–F). In contrast, no effects on chromosome morphology were observed when antibodies directed against tubulin, centrosomes, actin, or vimentin, were utilized or when chains were incubated with secondary antibodies alone for extended times (not shown). Furthermore, his-

Fig. 3. Phase contrast (**A–N**) and fluorescence micrographs (**O**) demonstrating structural memory in microsurgically removed nucleoplasmic strands. A–G: A mitotic strand (A) that was induced to undergo three repeated cycles of decondensation (B,D,F) and recondensation (C,E,G) by adding three consecutive 1 ul drops of 500 mM $MgCl_2$ directly above the strand. Arrows indicate one chromosome that repetitively disappears and reappears with the same form and position. H–J: An interphase strand (H) that also decondensed (I) and recondensed (J) in response to similar magnesium addition. Note that the large nucleolus at the middle left portion of the strand did not completely decondense in this experiment. K,L: While short term (20 min) exposure of this mitotic strand to dithiothreitol (10 mM) did not visibly alter native strand morphology (K), irreversible loss of chromosome form and genome pattern resulted (L) when strand decondensation was induced by addition of magnesium. M–O: Harpooning chromosomes in the presence of the DNA-binding dye, ethidium bromide (1–5 μg/ml final), resulted in removal of a distorted thread that lacked discrete chromosome forms (M). Release of tension on the pipette revealed loss of elasticity as well, as indicated by the failure of the strand to shorten and return back to its original retracted form (N). Fluorescence view of the same strand containing bound ethidium bromide demonstrating the loss of normal chromosome morphology within this distorted strand (O). (A–G, K–O: ×1200; H–J: ×900.)

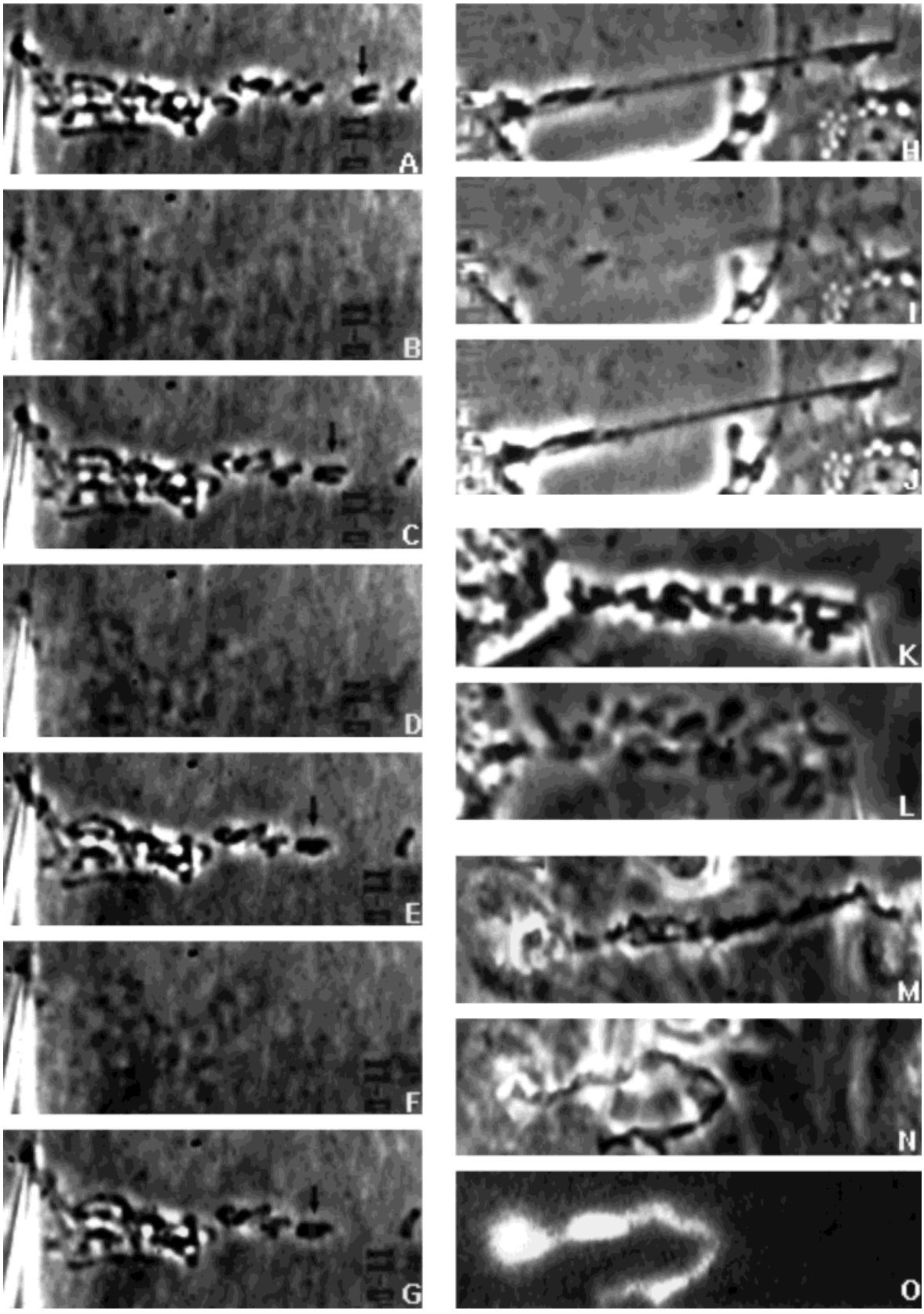

Figure 3.

tone antibody-condensed chromosomes were insensitive to ionic disruption, even in the presence of high salt (1–2 M NaCl).

In order to characterize the role of specific histones in genomic patterning, strands were treated with proteases (trypsin, 5 mg/ml; proteinase K, 50 ug/ml) or heparin (5 mg/ml), which have been reported to release these proteins from chromatin [Hacques et al., 1990; Hildebrand et al., 1977]. This treatment resulted in a rapid decondensation of chromosome chains, and the formation of a swollen cloud of DNA (Fig. 4G–I) that could not be reversed to its original pattern by varying cation concentrations (Table I). However, when histone H1 protein (1 ug/1 ul) was added to mitotic strands that were decondensed with either protease or heparin, rapid and precise reformation of chromosome chains occurred (Fig. 4J–L; Table I). This transformation resulted in restoration of original chromosome form as well as correct repositioning, such that most of the chromosomes returned to their pre-existing spatial locations relative to one another and to the entire genome (Fig. 4J vs. L). Furthermore, histone H1 also restored ionic sensitivity of the chains, such that they could decondense and recondense upon treatments with millimolar concentrations of cations. In contrast, the histones H2b and H3 were incapable of inducing recondensation or reconstitution even when added at 10-fold higher concentrations (H2b = 7/7; H3 = 5/5). Other proteins (e.g., BSA, tubulin, actin, heavy meromyosin) also did not alter the decondensed state of the proteolyzed or heparin-treated chromosomes (not shown).

DISCUSSION

Little is known about how chromosome movements are coordinated in time and space or how chromatin is organized and integrated with the cytoskeleton in living mammalian cells. Although the prevailing viewpoint regarding the organization of the genome is that it is composed of physically discrete and mechanically discontinuous chromosomal units, there is increasing evidence to suggest that a continuous anastomosing network of chromatin and non-chromatin fibers provide structural continuity during interphase as well as mitosis [Fey et al., 1984; Nickerson et al., 1992; Pickett-Heaps et al., 1984; Yin and Forer, 1996; Maniotis et al., 1997]. However, it is not clear if these conflicting interpretations result from differences in techniques, species differences, or variations in the cell culture conditions being utilized. Furthermore, when discontinuity is observed, artifactual breakage must be ruled out [Kavenoff and Zimm, 1973], and if continuity is observed, non-specific adhesions must be shown not to be the cause [Korf and Diakumacos, 1980].

In the present study, we developed a technique which permits us to directly test the extent of mechanical coupling between different nuclear structures within intact chromatin microsurgically removed from living endothelial cells at any moment during the cell cycle. In addition, we discovered that this method allows us to analyze the molecular basis of chromatin disassembly and reassembly within the intact genome under controlled conditions in vitro. Using this approach, we showed that under isotonic conditions, the native human or bovine endothelial cell genome behaves as a continuous, elastic structure. Based on a number of different results, this continuity did not appear to be the result of technical artifact: 1) every chromosome seen in more than 1,000 consecutive pulls was interconnected during rapid removal in the presence or absence of colchicine; 2) when tension was reapplied to a mitotic chain after it was allowed to collapse into an indistinct clump of chromatin, all of the indi-

Fig. 4. In situ analysis of the role of histones in chromosome recondensation. **A–C:** The discrete, phase-dense chromosome forms present within a mitotic chain immediately after microsurgical removal (A) disappeared when the concentration of $MgCl_2$ in the medium was raised to 50 mM (B) and then reappeared when a monoclonal antibody against histones (1;300 dilution; Chemicon) was subsequently added (C). **D–F:** Computerized densitometric quantitation of grey values measured along the main axis of the mitotic chains in A–C which clearly delineates the size and position of nine different chromosomes (**D**) that decondensed and became phase-lucent when the cation concentration was raised (**E**) and then reformed when histone antibodies were added (**F**). Integration of the area under the curves using an Oncor image analysis system revealed that the anti-histone antibodies restored greater than 95% of the phase contrast density exhibited by the original chromosome chain. **G–I:** Addition of trypsin (5 mg/ul drop) to a chromosome strand that was extended by pulling downward on the pipette shown at the bottom of the view in (G) resulted in complete loss of genome form (H) and release of disorganized DNA, as shown by staining with a DNA-binding fluorochrome, Yo-Yo (I). **J–L:** Trypsin treatment of the mitotic strand shown in (F) resulted in complete disappearance of the chromosome chain (**K**) which could be almost fully reversed by addition of histone H1 (1 ul drop of 1 mg/ml) as shown in (**L**). Arrows point to three examples of different chromosomes that were restored to their original form and position. (A–C, G–L: ×1000.)

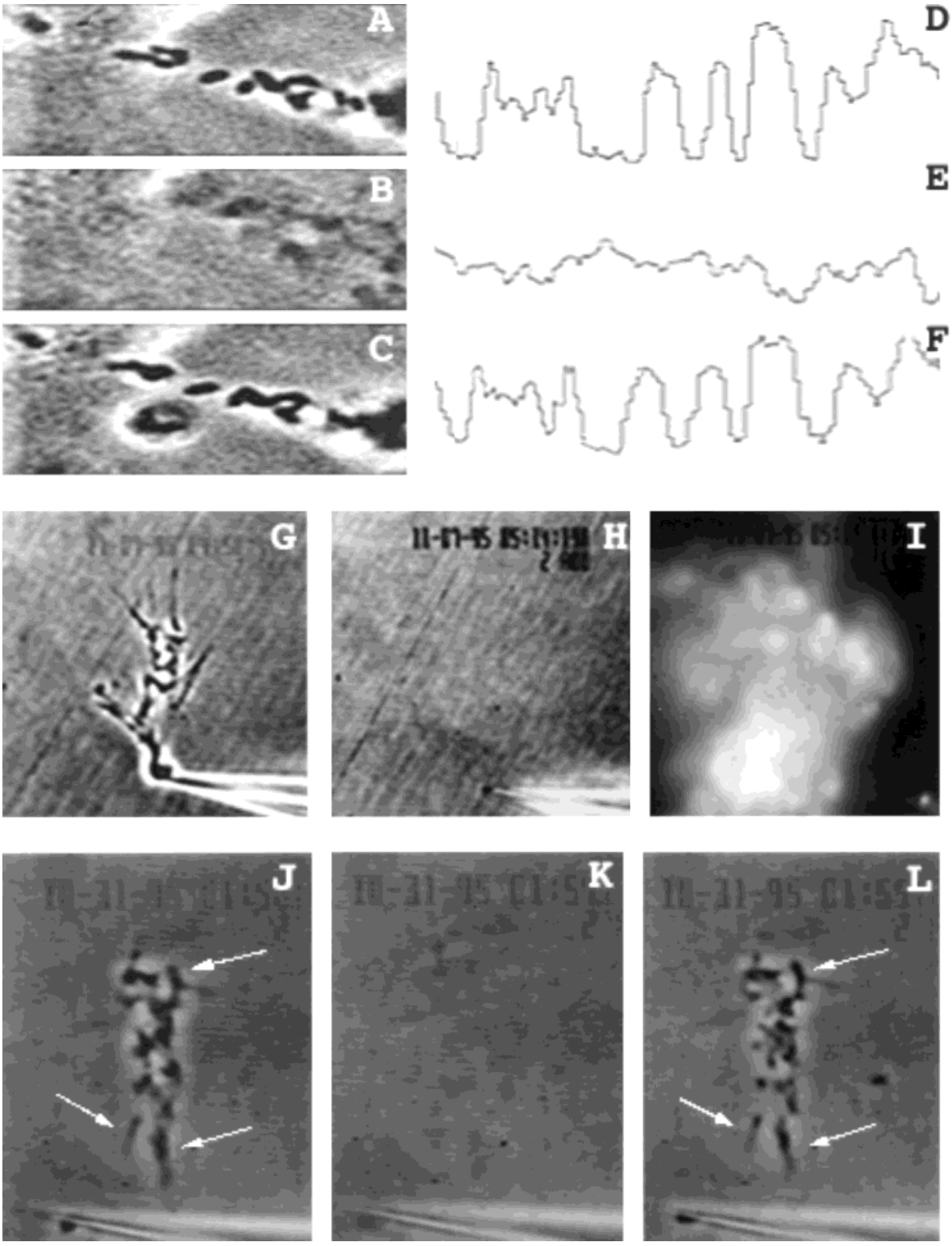

Figure 4.

vidual chromosomes separated and returned to their own pre-existing shape and position along the re-extended chain; 3) we could not intentionally force chromosomes to stick together by bringing them in direct juxtaposition; 4) when isolated chains were treated with heat and formamide, high ionic strengths, or proteases, continuity remained intact; and 5) we did not observe spatially separated chromosomes colliding in situ when we harpooned an individual chromosome, nor did they collide along the chain during removal (because they were always under tension). Furthermore, chromosomes do not normally stick or fuse together even though they may collide inside the living cell [Nicklas, personal communication]. This long-distance structural coupling may, in part, explain how multiple chromosomes and even entire chromosome plates can be moved as single entities without artifactual or accidental fusion of chromosomes, as previously described [Mazia, 1961; Nagele et al., 1995].

The physiological relevance of these connections is further supported by the ability of isolated mitotic chains to undergo dynamic and reversible structural transformations in a temporally and spatially coordinated manner. For example, when a 1 ul droplet of 50–500 mM NaCl or $MgCl_2$ was added to a removed chain, the chromosomes and intervening threads were able to fully decondense and then spontaneously regenerate their preexisting form as the ions diluted in the surrounding medium. The resolution of this memory was impressive, for example, individual chromosomes consistently restored their initial shape and position in the chain upon reversal of the ionic concentration and no stickiness or fusion was ever observed (Fig. 3A–G). Also, it has been established that microinjection of proteins such as tubulin [Mitchison et al., 1986] or calmodulin [Stemple et al., 1988] into mitotic mammalian cells does not interfere with normal mitotic segregation. Taken together, it therefore seems unlikely that changes in ion concentrations or other parameters due to micropuncture or microsurgical removal of the chains could be the cause of the connections we observed interlinking different chromosomes in this present study.

Past studies with isolated chromatin have shown that raising cation concentrations from micromolar to the millimolar range induces loosely organized 10 nm nucleosomal filaments to "condense" into tightly coiled 30 nm solenoid fibers [Thoma et al., 1979] analogous to those seen in native chromatin within intact chromosomes and interphase nuclei [Manuelidis, 1990; Pienta et al., 1991; Belmont and Bruce, 1994]. Given that we pull the nucleoplasm out into isotonic culture medium containing millimolar concentrations of cations, it is not surprising that both interphase and mitotic strands initially exhibited a condensed form consistent with the presence of well-packed solenoid fibers. However, our results demonstrate that chromatin packing is reversible and biphasic in its sensitivity to cations since native nucleoplasm containing condensed chromatin reversibly decondensed when exposed to additional cations. Similar changes in ion concentrations within local regions of the nucleus could regulate decondensation and condensation in vivo by either preventing or supporting release of coiled DNA and thereby, control its ability to unravel within distinct microenvironments.

The possibility that extended nucleoplasm and mitotic chromosome chains are connected and stabilized by DNA is suggested by the sensitivity of interphase and mitotic chains to DNase and micrococcal nuclease. Furthermore, mitotic threads (but not nucleoplasmic threads) were disrupted by restriction enzymes if they were first decondensed with ions and allowed to spontaneously recondense, reflecting perhaps the inability of these enzymes to access the sensitive DNA sites within the condensed chains, or the need for ion-induced changes in conformation to unravel the DNA after restriction enzyme sensitive sites are nicked (Table I). The finding that DNA binding dyes stained the interchromosomal connectives (Fig. 1Q) and altered the mechanical properties of the entire mitotic strand adds further support to the concept that the interchromosomal threads actually contain DNA, and that DNA is functioning as a fundamental mechanical element in the nucleus. While ethidium bromide was reported to have little direct effect on the elasticity of the isolated DNA helix [Smith et al., 1992], it can relax torsionally stressed DNA within extracted chromatin [Vogelstein et al., 1980]. Also, it is known that this dye can prevent the DNA helix from interconverting between its elongated and condensed forms [Smith et al., 1992; Cluzel et al., 1996]. Furthermore, attachment of chromatin loops to nuclear matrix is mediated by specific AT rich DNA sequences within scaffold attachment regions [Kas et al., 1993]. DNA

intercalators such as ethidium bromide and Hoechst 33258 also preferentially bind to these sequences, and thus they may alter strand mechanics and influence the pattern integrity of the entire genome through multiple mechanisms.

Taken together, these findings strongly suggest that DNA makes an important contribution to mechanical continuity both in interphase and mitosis. Furthermore, connectivity by DNA may be functionally important because it is known that telomeric DNA can form a variety of non-Watson-Crick base-paired structures, including four-stranded helices or G quartets [Sen and Gilbert, 1988; Williamson et al., 1989; Henderson et al., 1987]. Thus, it is possible that telomere ends of different chromosomes, which play a critical role in defining long-term cell survival (cellular senescence), could interconnect through these novel base pairing regions in vivo.

On the other hand, mitotic strands that were treated with restriction endonucleases could withstand a single round of decondensation and recondensation by adding magnesium. Breakage only resulted when a second round of decondensation was induced. Homopolymers of histone H1 present within native chromatin could prevent initial strand breakage since they have been reported to maintain nucleosomal strands in their condensed form even in the presence of discontinuities in the DNA helix [Ruiz-Carrillo et al., 1980]. Nuclear matrix scaffolds that interconnect different chromosomes [Nickerson et al., 1992] also could contribute to this response. However, these protein-based interactions apparently can not withstand repeated deformation in the absence of intact DNA.

The finding that ion-decondensed genomes can be induced to recondense into chromosome chains by addition of a monoclonal antibody against histones suggests that histone-histone interactions also play a key role in the patterning of mitotic chromosomes. Histone H1 appeared to be most critical in this context since only this histone, and not Histones H2B or H3 or other proteins (Table I), could rescue chromosome pattern and position in this model system. These results point to the possibility that, in addition to its established role in compacting the DNA into characteristic 30 nanometer coils, histone H1 polymerization or aggregation may also drive higher order structural transformations that pattern the entire genome. This concept conflicts, however, with results obtained with a variant form of histone H1, called H1X, that is found only in xenopus eggs [Ohsumi et al., 1993]. Immunodepletion of H1X from Xenopus extracts does not interfere with chromosome condensation, although the resulting chromosomes do appear to be more fragile, suggesting that this form of histone H1 may be important for mechanical stability but not for condensation. Thus, the role of histone H1 in chromosome assembly and genomic organization may vary between different cell systems.

While purified histone H1 can induce isolated DNA to condense into nucleosomal fibers, it does not create chromosomes in vitro [Thoma et al., 1979]. Non-histone scaffolding proteins therefore also must be present to reconstitute full genome architecture, including generation of intact chromosomes with characteristic form and position, as previously suggested [Pienta et al., 1991; Paulson and Laemmli, 1977]. In addition, our studies with native nucleoplasm revealed dynamic qualities of the nuclear protein matrix that were previously absent in extracted or fixed preparations. Namely, these disulfide-stabilized nuclear scaffolds were required for maintenance of pattern integrity during reversible changes in higher order genome architecture. The nuclear matrix also regulates nuclear physiology by defining boundaries to DNA unwinding, providing a mechanical scaffold to resist torsional stress, and bringing relevant enzymes and structural proteins (e.g., DNA polymerase, topoisomerases, RNA polymerases, histones) into close proximity to their site of action on the DNA helix [Vogelstein et al., 1980; Kas et al., 1989; Stief et al., 1989; Manuelidis, 1990; Pienta et al., 1991]. By forming a composite material with nuclear scaffolding proteins, chromatin is therefore able to provide the flexibility and form stability necessary for nuclear organization as well as the functional diversity required for control of growth and gene expression. Thus, while DNA may provide chromatin with its mechanical integrity and histone H1 enables its to undergo dynamic structural transformations, the disulfide-stabilized nuclear protein matrix appears to stabilize higher order chromosome architecture and to preserve pattern integrity within the genome.

In summary, changes in chromatin organization and condensation play a central role in

transcription, DNA replication, recombination, and repair as well as in the transition between interphase and mitosis, yet it remains unclear how these structural alterations take place in living cells. We conclude from our studies that the entire genome functions as a structurally unified system. Chromatin self-stabilizes its form through establishment of a local force balance: expansive forces contained with mechanically constrained regions of coiled DNA (i.e., analogous to springs) resist equal and opposite inward-directed (compressive) forces imposed by interconnected histone H1 homopolymers [Thoma et al., 1979]. In fact, overcompaction and stiffness could be demonstrated directly in our system by adding excess histone H1 to native mitotic strands; this resulted in overcondensation (by up to 140% as detected using computerized image analysis) and loss of elasticity of the whole genome (n = 6/6). Other disulfide-linked nuclear protein scaffolds, in turn, guide how these stiffened coils fold into higher order structures with specific forms, such as mitotic chromosomes, interchromosomal threads, or interphase nucleoplasm. In this manner, the degree of compaction and stiffness of chromosomes may be controlled locally in situ, as suggested by analysis of high resolution deconvolution images of chromatin in early G1 [Belmont et al., 1994]. However, DNA itself appears to provide structural continuity throughout the entire genome and, when mechanically constrained by histone H1, the potential energy that can be harnessed to drive structural interconversions within these different nucleoplasmic conformations.

In interphase cells, the nucleoplasm is further stabilized through formation of multiple discrete connections with the surrounding nuclear lamina [Fey et al., 1984; Maniotis et al., 1997] whereas the chromosome chain connects directly to cytoplasmic microfilaments in mitotic cell. In addition, the chromosome necklace interconnects with multiple microtubules in the mitotic spindle. By pushing out against this elastic chromatin network, the microtubule struts mechanically stiffen and align the entire spindle apparatus, much as the insertion of rigid poles in a nylon tent stiffens and orients its roof and walls. This type of architectural system that gains its mechanical stability and flexibility by using isolated compression struts to place a surrounding structural network under tension is known as tensegrity [Ingber et al., 1994]. A similar tensegrity-based mechanism involving cytoplasmic microtubule bundles, cross-linked actin bundles, and external tethers to extracellular matrix scaffolds act in a similar manner to stabilize the surrounding microfilament and intermediate filament lattice in an extended form [Ingber et al., 1994].

The stability of this type of prestressed spindle network that uses DNA as its principal tension element may serve to ensure fidelity of chromosome segregation during mitosis and thus, genomic stability, by ordering chromosome movements in space. Hard-wiring within the nucleus also provides a physical basis to explain how precise spatial positioning of individual chromosomes can be maintained [Nagele et al., 1995] as well as how the movement of one chromosome can influence the timing and movements of other chromosomes in the same mitotic spindle [Yin and Forer, 1996; Li and Nicklas, 1995; Reider and Alexander, 1991]. In addition, the elastic forces stored within this tensed chromatin network could work in concert with microtubule depolymerization (i.e., strut shortening) to move chromosomes toward the spindle poles [Ingber et al., 1994]. In cells with discontinuous chromosomes (e.g., certain insect cells), the interconnected tensile actin network may provide these motive forces instead of DNA [Forer, 1966; Yin and Forer, 1996]. This may explain why cortical actin filaments become stretched parallel to the spindle axis during poleward movement of mitotic chromosomes in certain cells [Schmit and Lamber, 1987; Maro et al., 1986].

Thus, while mechanical stabilization of the mitotic apparatus may be mediated by different physical elements in different cell types, regulation of the timing of chromosome separation and of genomic stability may be similarly achieved through maintenance of an internal tensegrity force balance. Furthermore, tensile forces transmitted across cytoskeletal interconnections within this structurally unified system provide an alternative source of force generation (i.e., separate from microtubule polymerization) that may explain why certain chromosome movements can proceed in the absence of intact microtubules [Forer, 1966; Pickett-Heaps et al., 1984], how distant chromosomes can influence each other's movements independently of the spindle apparatus [Yin and Forer et al., 1996], and how mechanical stresses applied to the cell surface can result in

mitotic spindle reorientation in plants [Lintilhac and Vesecky, 1984] as well as animal cells [Maniotis et al., 1997]. Continuity and dynamic plasticity within the genome and surrounding cytoskeleton may therefore provide a mechanical basis for integrating structure and function within cells and nuclei throughout the cell cycle.

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