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DNA Topoisomerase II Can Drive Changes in Higher Order Chromosome Architecture Without Enzymatically Modifying DNA

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Abstract Topoisomerase II has been suggested to play a major role in chromosome organization based on its DNA decatenating activity and its ability to mediate direct binding interactions between DNA and nuclear matrix. However, this latter point remains controversial. Here we address the question of whether the chromatin binding activity of Topoisomerase II is sufficient to modify chromosome form using whole mammalian chromosomes in vitro. Intact chromosomes were microsurgically removed from living cells and disassembled by treatment with protease or heparin. When these disassembled chromosomes were incubated with recombinant human Topoisomerase II, the enzyme became incorporated into chromatin and reassembly resulted, leading to almost complete restoration of pre-existing chromosome shape and position within minutes. Chromosome reconstituition by Topoisomerase II was dose-dependent, saturable, and appeared to be controlled stoichiometrically, rather than enzymatically. Similar reassembly was observed in the absence of ATP and when a catalytically inactive thermosensitive Topoisomerase II mutant was used at the restrictive temperature. Chromosome recondensation also could be induced after the strand-passing activity of Topoisomerase II was blocked by treatment with an inhibitor of its catalytic activity, amsacrine. When a non-hydrolyzable β,γ-imido analog of ATP (AMP-PNP) was used to physiologically fix bound Topoisomerase II enzyme in a closed form around DNA, subsequent chromosome disassembly was prevented in the presence of high salt. These data suggest that Topoisomerase II may control higher order chromatin architecture through direct binding interactions, independently of its well-known catalytic activity. J. Cell. Biochem. 69:127–142, 1998.

Key words: chromosome architecture; disassembly; reassembly; proteases; in vitro model

DNA topoisomerase II (Topo II) is a nuclear mechanoenzyme responsible for controlling DNA topology. The catalytic activity of this homodimeric protein involves ATP-dependent passage of a double-stranded DNA segment through a transient break generated in a second double-stranded DNA segment, resulting in its catenation, decatenation, or relaxation [Osheroff et al., 1983]. The catalytic activity of Topo II has been shown to be required for the segregation of sister chromatids during the metaphase-anaphase transition [Uemura et al., 1987; Holm et al., 1989; Shamu and Murray, 1992; Clarke et al., 1993]. However, inactivation of Topo II prior to condensation only partially inhibits chromosome compaction [Uemura et al., 1987], suggesting that the catalytic activity of Topo II cannot entirely account for control of higher order chromosome architecture.

One possibility is that Topo II also plays a structural role in chromosome organization, independent of its catalytic activity. Topo II is one of the most abundant components of the insoluble nuclear matrix scaffold that is thought to form the molecular backbone of chromosomes [Earnshaw et al., 1985; Gasser et al., 1986]. Furthermore, Topo II preferentially localizes to the matrix-associated regions of DNA, through which DNA loops attach to the chromosome scaffold [Adachi et al., 1989]. Thus, nuclear matrix-associated Topo II has been proposed to

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both physically fasten chromatin loops to the insoluble chromosome scaffold and to regulate DNA topology through its catalytic activity. However, the relevance of Topo II's direct binding interactions is questionable given that most of Topo II can be dissociated from chromatin without altering chromosome morphology [Hirano and Mitchison, 1993; Swedlow et al., 1993]. Chromosome architecture also is not changed by treatment of cells arrested in mitosis with inhibitors of Topo II catalytic activity. Thus, the conventional view is that Topo II is needed for the establishment but not for the maintenance of condensed chromosome form [Koshland and Strunnikov, 1996]. Still, it is possible that the subset of Topo II that always remains tightly associated with chromatin can control chromosome architecture through direct protein binding interactions, independently of its strand-passing catalytic activity.

We, therefore, set out to determine whether Topo II can modify chromatin architecture through direct protein binding interactions within whole chromosomes in vitro. It has not been possible to address this question in mammalian cells in the past due to the lack of a model system that permits direct analysis of chromosome structure and dynamics in vitro. In the present study, we capitalized on the recent development of a microsurgical technique that results in the removal of multiple intact metaphase chromosomes from living endothelial cells [Maniotis et al., 1997]. Chromosomes isolated using this method undergo disassembly when treated with protease or heparin to remove nuclear proteins. Importantly, the proteolyzed (disassembled) chromosomes retain the ability to reassemble when specific nuclear proteins (e.g., histone H1 protein but not H2B or H3) are added, resulting in nearly complete restoration of preexisting chromosome size, shape, and position [Maniotis et al., 1997]. In the present study, we use these disassembled mammalian chromosomes to analyze the effect of recombinant Topo II enzyme on higher order chromosome architecture under defined conditions in vitro.

MATERIALS AND METHODS

In Vitro System for Analysis of Chromosome Architecture

Bovine capillary endothelial cells were grown on glass coverslips (VWR Scientific, Boston, MA) in bicarbonate-buffered medium containing DMEM supplemented with 10% calf serum and 5 ng/ml fibroblast growth factor, as previously described [Ingber, 1990]. Immediately prior to microsurgical manipulation, the coverslips and adherent cells were transferred to 35-mm Petri dishes (Falcon, Lincoln Park, NJ; Becton Dickinson, San Jose, CA) and placed in 2 ml of culture medium lacking serum and growth factors. Chromosome isolation and manipulation were performed in serum-free culture medium (DMEM with 10 mM HEPES, pH 7.4) on a Nikon Diaphot inverted microscope fitted with a 60× long working distance objective. Isolation of chromosomes was performed as described by Maniotis et al. [1997]. In brief, intact chromosomes were microsurgically removed from living mitotic cells by rapidly piercing ("harpooning") the cell with a fine glass micropipette, touching a single chromosome and then laterally drawing out the attached chromosome using a Leitz (Wetzlar, Germany) micromanipulator. Pulling out on a single chromosome in this manner resulted in progressive removal of all of the remaining chromosomes as if they were beads interconnected by a continuous thread (Fig. 1A). This effect was not due to cell death or generalized toxicity since harpooned cells continued to proceed through mitosis and divide [see Maniotis et al., 1997, for a further description of controls and specificity]. Because the protease treatment used in certain studies caused cell detachment, entire extended chromosome chains were completely removed and physically attached to a free area of the culture substrate by gently touching the removed chromosome chain to the surface prior to exposure to enzyme; all manipulations were carried out in the absence of serum. Micropipettes were pulled with a Sutter micropipette puller adjusted to produce tip widths less than 0.5 μm.

Disassembly of isolated chromosomes was produced by adding 10 μl of a protease mixture containing trypsin (5 mg/ml; Gibco, Grand Island, NY) and proteinase K (0.25 mg/ml; Boehringer Mannheim, Indianapolis, IN) or 1 μl of a heparin solution (10 mg/ml; Sigma, St. Louis, MO) directly above the isolated chromosomes (final volume = 2 ml). Chromatin disassembly was allowed to proceed for 3–5 min under direct microscopic observation, until near-complete loss of detectable phase contrast density occurred. Chromosomes were washed two times

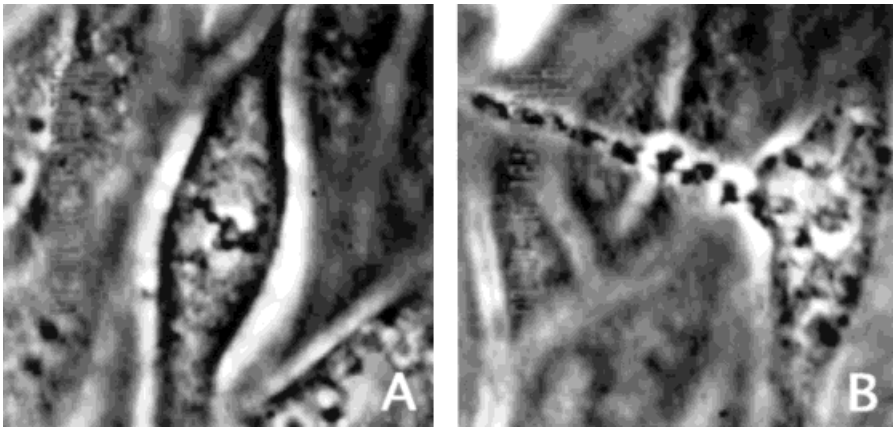

Fig. 1. Microsurgical isolation of chromosomes from a living mitotic endothelial cell. **A:** A phase contrast view of a metaphase chromosome plate within a mitotic endothelial cell. **B:** A chromosome chain microsurgically removed from the cell shown in A after harpooning with a glass micropipette. The pipette tip can be seen adherent to the first chromosome of the extended chain at the top left of this view (×1,600).

with 2 ml of buffered DMEM and analyzed after addition of fresh serum-free medium.

Reassembly of proteolyzed or heparinized chromosomes was induced by washing twice with 2 ml DMEM, adding 1-2 µl of purified Topo II (1 µg/µl) directly above the disassembled preparation, and monitoring chromosome recondensation using phase video microscopy. Other compounds used to test their effect on chromatin reassembly included spermine (10 µg, Sigma), Topoisomerase I (2 µg), HIV-1 integrase (6 µg, Intracel, Seattle, WA), Histone H1 (2 µg, Sigma). To examine the role of Topo II enzyme activity, 200 µM m-AMSA (4-[9-acridinilamino]-N-[methanesulfonyl]-m-anisidine; Sigma) was included in the reconstitution medium with the purified enzyme. To analyze the ability of exogenous Topo II to interact with DNA, Topo II-reconstituted chromosomes were incubated with 2.5 mM AMP-PNP (5′-adenylylimidodiphosphate; Sigma) in DMEM containing 8 mM $MgCl_2$ for 10 min at room temperature.

Microscopy and Image Analysis

Phase contrast images were recorded using real-time videomicroscopy on a Nikon Diaphot microscope connected to DAGE MTI camera and GYRR videotape recorder. Recorded images were digitized and phase contrast pixel densities analyzed using a Macintosh Quadra 800 computer and Oncor (Gaithersburg, MD) image analysis software. Images were converted to PICT format for presentation using Adobe Photoshop software.

Immunofluorescence staining of Topo II-reconstituted chromosome chains was carried out by incubating unfixed chromosome chains for 1 hr in the presence of rabbit polyclonal antibodies against human Topo II (dilution 1/30 in DMEM; Topogen, Columbus, OH). After washing three times in DMEM, secondary donkey anti-rabbit IgG (Texas red-linked, dilution 1/40 in DMEM; Amersham, Arlington Heights, IL) was applied for an additional hour and then washed three times in DMEM. Other probes used included antibodies specifically directed against histone H1 (Biogenex, San Ramon, CA; 1/50) or against total histones (1/100; Chemicon, Temecula, CA). The unfixed chains were immediately observed under oil immersion and images recorded on a Zeiss (Thornwood, NY) Axiophot photomicroscope.

DNA was visualized by adding 4,6-Diamidino-2-phenylindole (DAPI) or Propidium Iodide (Sigma) directly to the medium (0.3 µg/ml final concentration). Alternatively, microsurgically isolated chromosomes were stained in situ using a quinacrine banding protocol. In brief, intact adherent human endothelial cells were pre-incubated in colcemid (10 µg/ml) for 30 min, exposed to hypotonic conditions by adding distilled water (approximately 35 mM final salt concentration) for 24 min, followed by several changes of 3:1 methanol/acetic acid solution, drying under humidified conditions, baking for 2 hr at 80°C, and staining with quinacrine (20 mg/ml; Sigma) for 7 min. The specimens are washed in McIlvaine's solution (pH 5.5) for 20 s

and then mounted in dilute McIlvaine's solution. In experiments with surgically removed chains, cells were pretreated with 10 µg/ml colcemid for 30 min without osmotic swelling before removal. In reconstitution studies, the chromosome chains removed from colcemid-treated cells were exposed to heparin, washed with DMEM, and induced to resassemble by addition of Topo II.

Purification of Topoisomerase I and II

Recombinant human Topo II (topoisomerase IIα) was purified as reported [Worland and Wang, 1989]. Briefly, Topo II was overexpressed from a multicopy expression plasmid YEpWob6 in *Saccharomyces cerevisiae* [Wasserman et al., 1993] and purified by a four-step procedure consisting of yeast disruption, elution from polyethyleneimine/Celite column, ammonium sulfate precipitation, and Phosphoultragel chromatography. Purified Topo II was dialyzed against the storage buffer (50 mM Tris-HCl, pH 7.7, 100 mM KCl, 1 mM EDTA, 1 mM EGTA, 50% glycerol) and stored at −70°C. Topo II aliquots were dialyzed against PBS for 35 min (VS 0.025 µm filters; Millipore, Bedford, MA) immediately before use.

Wild type yeast Topoisomerase II and the temperature-sensitive top2-5 mutant were generously provided by Dr. John L. Nitiss (Memphis, TN). Wild type and mutant proteins were overexpressed from multicopy plasmids under GAL1 promoter in JN362a yeast strain and purified as previously described [Worland and Wang, 1989]. Recombinant Topoisomerase I was purified from infected Sf21 cells, as previously described [Rossi et al., 1996].

Topoisomerase II Catalytic Assay

Topo II catalytic activity was assayed by decatenation of kinetoplast DNA (kDNA; Topogen). Reaction mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 165 mM KCl, and 150 ng kDNA. When ATP was included, its concentration was 1 mM. Reactions were initiated by addition of Topo II, incubated for 6 min at 37°C, and terminated by addition of SDS (1%)-bromophenol blue(0.05%)-sucrose(10%, final concentrations). Samples were electrophoresed at 90 V for 2 hr on 1% agarose gel in Tris/Acetate/EDTA buffer. Gels were stained in 0.5 µg/ml ethidium bromide and photographed under UV light illumination.

RESULTS

Purified Topo II Induces Chromosome Reassembly In Vitro

Intact chromosomes were microsurgically removed from living endothelial cells by harpooning the metaphase nucleus with an ultrathin glass micropipette (tip less than 0.5 µm wide) and pulling outward on a single chromosome (Fig. 1). This procedure resulted in removal of multiple chromosomes interconnected by a continuous thread, as previously described [Maniotis et al., 1997]. To facilitate morphological analysis, the extended chromosome chain was tacked down onto the culture dish before chromosome disassembly was induced (Fig. 2A) by gently pushing the attached proximal end of the strand against the dish surface and then pulling the pipette away using the micromanipulator. Treatment of chromosomes with a mixture of general proteases (proteinase K and trypsin) that degrade key nuclear structural proteins [Weintraub and van Lente, 1974; Tsutsui et al., 1988; Hacques et al., 1990; Whitlock and Simpson, 1977; Lundell and Martinson, 1989] resulted in chromosome disassembly. These proteolyzed chromosomes can be induced to reassemble by addition of histone H1, but not other histone proteins [Maniotis et al., 1997]. This capability to drive large-scale, reversible transformations in chromosome structure, combined with the molecular specificity of this response, implied that this system may represent a useful model for analysis of chromosome structure and dynamics in vitro.

We, therefore, used this model to directly determine whether Topo II can interact with chromatin and modify chromosome form. When purified recombinant human Topo II was added to a chromosome chain that was disassembled (completely phase-lucent) due to proteolysis (Fig. 2B), progressive reassembly of chromatin resulted, leading to reformation of multiple distinct phase-dense chromosomes within 4 to 6 min (Fig. 2C,D). Immunofluorescence analysis with anti-human Topo II antibodies revealed bright staining of most of the reformed chromosomes from bovine cells (Fig. 2E). The chromosomes that failed to stain largely coincided with crushed areas of the strand that also failed to disassemble in response to protease treatment (Fig. 2D,E). These data confirm that the human

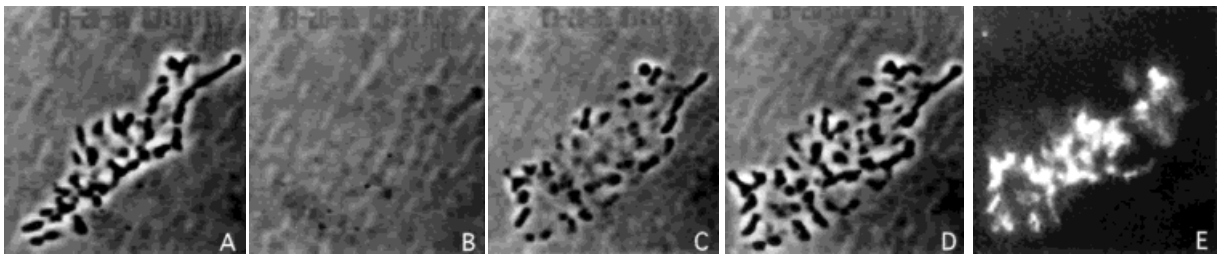

Fig. 2. Reassembly of protease-digested chromosomes by addition of purified Topo II protein. **A:** Phase contrast view of a chromosome chain after microsurgical removal and attachment to the culture dish surface. **B:** The same chain after exposure to a mixture of trypsin and proteinase K. Complete disassembly of the chromosome chain has resulted such that the chromatin strands are beyond the limits of visualization by phase-contrast microscopy. **C:** The same chain after being washed extensively and having purified Topo II added to the medium for 3 min. **D:** The same chain in the same medium 4 min later. These images show that protease-disassembled chromosomes progressively reassembled in response to exposure to exogenous Topo II, passing first through a partially condensed intermediate state (C), before completely reconstituting preexisting phase-dense chromosome forms (D). **E:** Immunofluorescence view of the same unfixed chain extracted from bovine cells, stained with anti-human Topo II antibodies. Positive staining for added (human) Topo II was observed within almost all of the reconstituted chromosomes. Note that the crushed chromosome at the top right of the cluster that failed to disassemble in response to protease treatment also stained negatively for Topo II (×1,125).

Topo II enzyme we added became physically associated with chromatin during the reassembly process. Similar results were obtained with yeast Topo II whereas addition of the Topo II buffer in the absence of enzyme had no effect on chromosome structure (data not shown).

We then used heparin to further analyze the fidelity of chromosome reassembly induced by Topo II. This compound chemically disrupts protein-DNA and protein-protein interactions and releases histones without causing protein degradation [Hildebrand et al., 1977; Strzelecka et al., 1983; Kassavetis et al., 1989; Bojanowski et al., 1993]. To avoid potential artifacts induced by the chromosomes adhering to the plastic substrates, the chromosome chain was attached to a solid support only at its two ends. One end of the chromosome strand remained naturally tethered to the structural elements of the cell and the other end, to the tip of the micropipette (Fig. 3A). When Topo II was added to chromosomes that were disassembled with heparin (Fig. 3A,B), nearly complete recovery of pre-existing chromosome morphology resulted (Fig. 3C). Computerized quantitation of changes in phase contrast densities (pixel densities) within these images confirmed that Topo II-reconstituted chromosomes recovered more than 90% of the original shape and degree of chromosome compaction (Fig. 3D–F).

It is possible that the Topo II we added binds to a residual scaffold of proteolyzed chromosomes and, thus, only gives the appearance of condensed chromosomes without actually rewinding the released loops of DNA. We, therefore, stained surgically removed native (Fig. 4A,B), proteolyzed (Fig. 4C,D), and Topo II reconstituted chromosomes (Fig. 4E,F) with the DNA-binding dye propidium iodide to examine how DNA organization changes under these different conditions. These experiments confirmed that proteolysis resulted in an explosive release of the normally, tightly packed DNA, which reversed upon treatment with Topo II. Reconstitution of preexisting chromosome morphology also could be demonstrated using a quinacrine staining methodology commonly used in chromosome "banding" techniques (Fig. 4G–I), even though this conventional staining process involves unphysiological osmotic swelling, fixation and dehydration steps. Taken together, these results clearly show that proteolysis resulted in complete loss of normal chromosome organization, which could be effectively reversed by exposure to purified Topo II protein.

Subsequently, we tested several other compounds that bind to DNA for their ability to reassemble chromatin, including spermine, a polyamine that has a strong affinity to DNA and plays an important role in the chromatin structure [for review see Matthews, 1993] and HIV-1 Integrase, which binds and reacts with DNA in a manner similar to Topo II [Carteau et al., 1993]. Spermine was incapable of inducing full chromosome reassembly; however, a transient condensation was observed that spontaneously reversed after a period of seconds to minutes (Fig. 5A–H). HIV-1 integrase did not produce any detectable condensation in our sys-

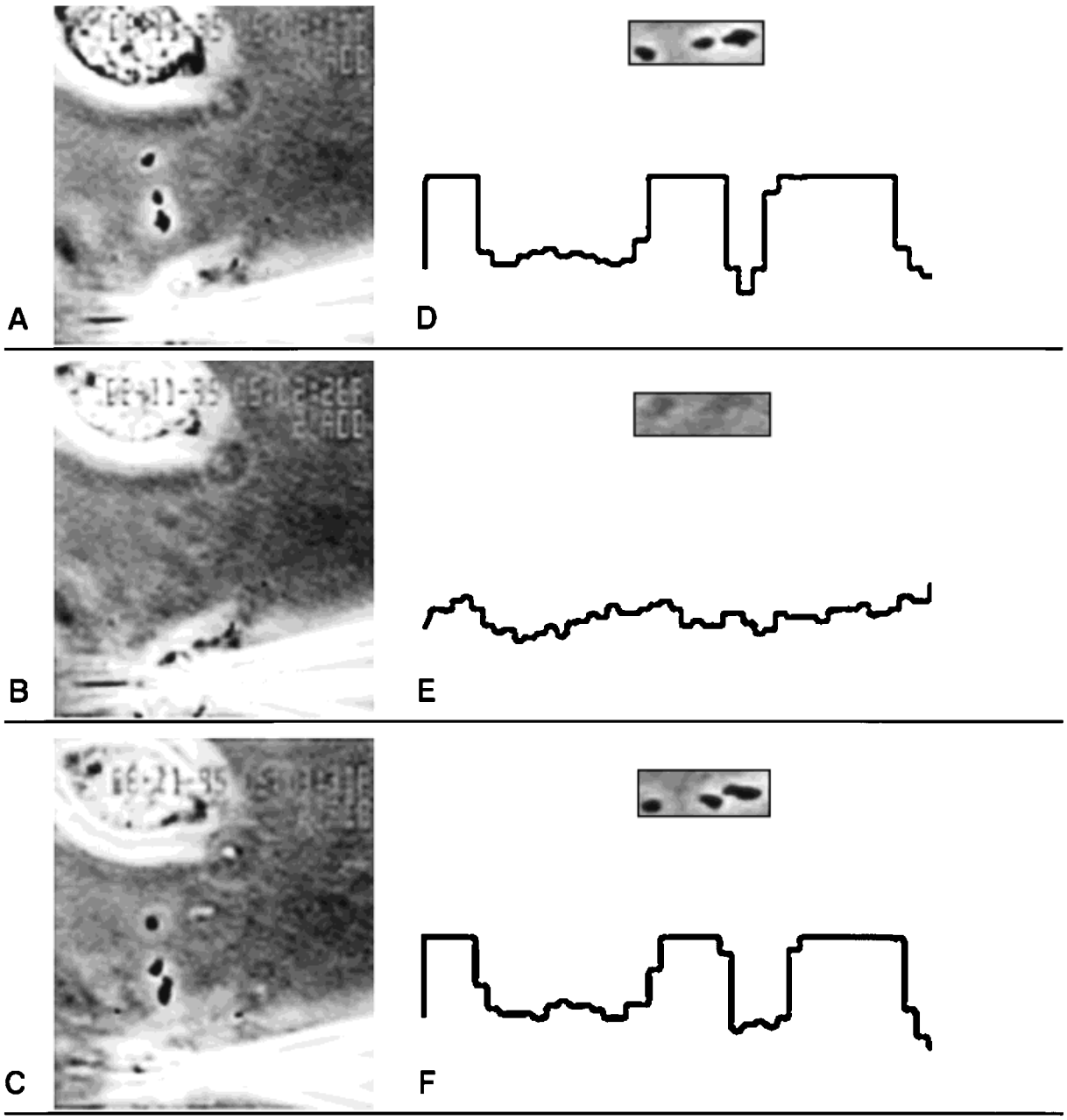

Fig. 3. Chromosome reassembly with purified Topo II within heparin-disassembled chromosomes. **A:** Phase contrast view of three adjacent chromosomes removed using the harpooning procedure; the largest chromosome at the bottom may represent two closely associated chromosomes. The pipette tip remains connected to the proximal end of the strand and is visible at the bottom of this view; the remaining chromosomes are still contained within the cell that appears at the top of the view. Between the two tethered ends of the chain, chromosomes are not in contact with any solid support. **B:** The same chain after disassembly by addition of heparin to the medium, demonstrating near complete loss of chromosome morphology. **C:** The same heparin-disassembled chain shown in B, 5 min after addition of purified Topo II. Almost full restoration of chromosome morphology and position was observed. **D–F:** Computerized quantitation of the phase contrast pixel density measured along a line drawn along the main horizontal axis of the extended chromosome chain when oriented as shown at the top. The graphs below depict the densitometric results and show the presence of the three chromosomal densities at the start, their complete loss after heparin treatment, and nearly complete reconstitution of the three chromosomes following Topo II addition in D, E, and F, respectively. In this study, heparin induced a loss of greater than 95% of the phase dense pixels in the region of the three chromosomes. Conversely, addition of Topo II resulted in restoration of more than 90% of the original pixel density in regions that corresponded precisely to those inhabited by the original untreated chromosomes (×1,600).

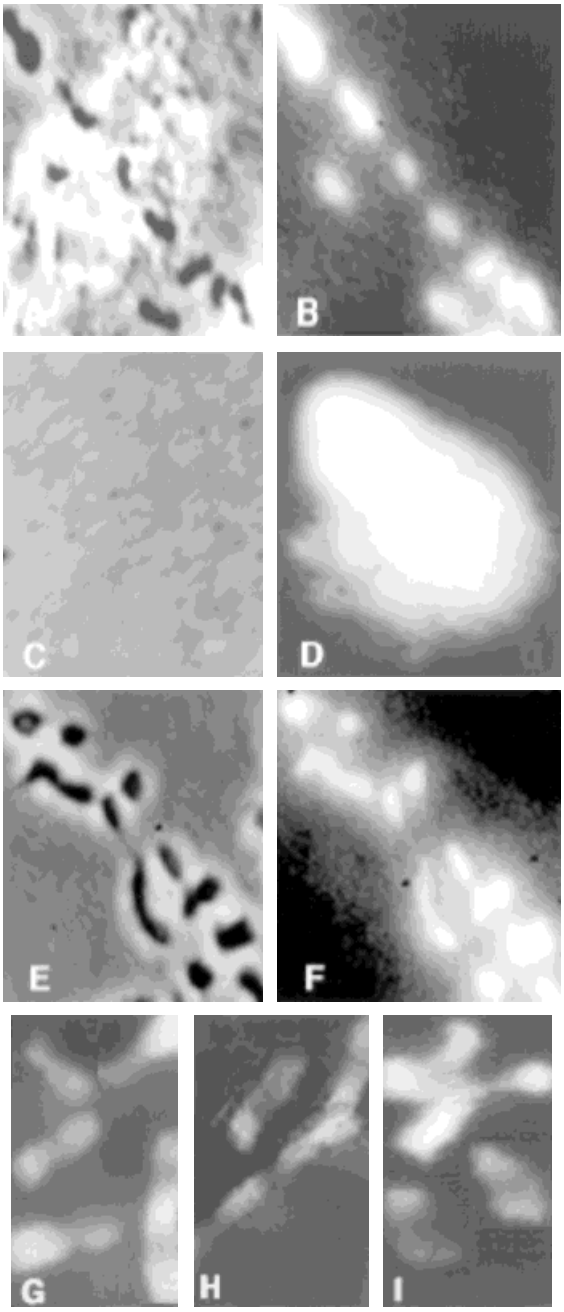

Fig. 4. Morphology of native (**A,B**), proteolyzed (**C,D**), and Topo II-reconstituted (**E,F**) chromosome chains visualized by phase contrast (A, C, E) or fluorescence microscopy in conjunction with propidium iodide staining (B, D, F) (×1,800). Similar results were obtained with DNA-binding dyes in more than 20 different experiments. Quinacrine staining pattern of chromosomes from an intact cell after osmotic swelling (**G**), an intact chain removed using microsurgery (**H**), or a reconstituted chain after heparin treatment and addition of Topo II (**I**) (×3,375). Distinct chromosome morphology, including the presence of paired homologues with primary metacentric constrictions, were visible in chromosomes under all three conditions. Only native chromosomes isolated from cells that underwent osmotic swelling displayed discrete banding patterns.

tem, although immunofluorescence analysis showed that it physically associated with the unfolded chromatin (Fig. 5 I–L). We then tested topoisomerase I (Topo I), a functionally related enzyme [for review see Wang, 1991], which also contains highly cationic, lysine-rich domains similar to those found in Topo II and histone H1. Topo I produced similar results as Topo II in this system (Fig. 6A,C vs. 6F,H), as did histone H1 (Fig. 6K,M). These results suggest that electrostatic interac-

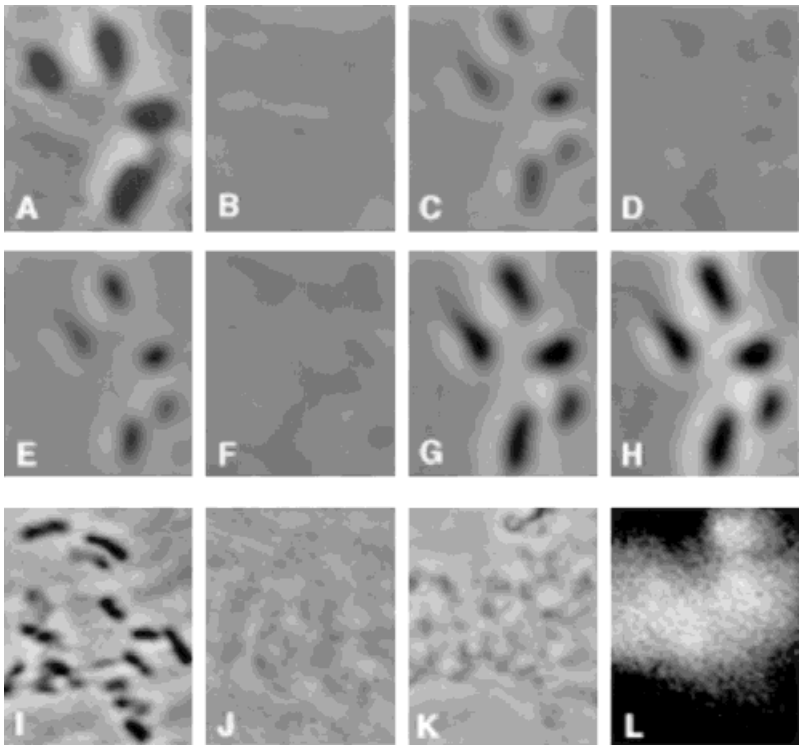

Fig. 5. Effect of spermine (**A–H;** ×2,600) and HIV-1 integrase (**I–L;** ×1,600) on the morphology of protease-treated chromosomes. Native chromosomes (A, I) were disassembled by protease (B, J) and incubated with 50 µmol spermine (C) or with 6 µg of fluroscent (FITC-labeled) HIV-1 integrase (K). Spermine induced partial chromosome reassembly within 2 min after addition (C), but this response was transient and spontaneously reversed by 4 min (D). Addition of more (50 µmol) spermine to the disassembled chain shown in D resulted in another transient wave of partial reassembly over the following 2 min (E,F). When these disassembled chromosomes were then incubated with Topo II (1 µg), complete reassembly of the chromosomes resulted (G), which remained stable for more than 1 hr after addition (H). Integrase bound to disassembled chromosomes (L); however, it did not induce chromosome reassembly (K,L).

tions play a key role in these large-scale chromosome restructuring events.

In order to gain molecular information regarding the composition of proteolyzed chains, we stained the chromosome chains reconstituted with Topo I, Topo II, or histone H1 with antibodies against all histones, histone H1, or Topo II (Fig. 6). Quantitation of fluorescence intensity of native, proteolyzed, and reconstituted chains revealed that, on avereage, proteolysis removed more than 75% of Topo II and histone H1 from the native chromatin, while the proteolyzed chromosomes continued to stain positively for core histones (compare Fig. 6B vs. D). Thus, residual chromatin proteins that remain tightly associated with the unwound DNA may guide the reassembly process driven by exogenous Topo II, Histone H1, or Topo I.

Topo II Does Not Require Its Catalytic Activity to Induce Chromosome Reassembly

The finding that Topo II can induce changes in higher order chromosome architecture in this in vitro model permitted us to ask directly whether its catalytic activity was required for this effect. The first suggestion that the effects of Topo II on chromosome reassembly might be independent of its enzyme activity was based on the observation that we did not include ATP in the incubation medium; ATP is required for the decatenating activity of Topo II [Osheroff et al., 1983]. Furthermore, we found that the effect of Topo II on chromosome reassembly was controlled stoichiometrically, rather than catalytically. For example, when proteolyzed chromosomes (Fig. 7A,B) were incubated with a suboptimal amount of Topo II (0.8 µg/4 µl droplet added to 2 ml final volume), only partial recondensation was observed (Fig. 7C), even after prolonged incubation times or after addition of ATP and magnesium (not shown). Addition of another similar dose of Topo II resulted in a step-like increase in chromosome reassembly, which again did not progress with time of exposure (Fig. 7D). Addition of a third dose of Topo II resulted in an additional increase in

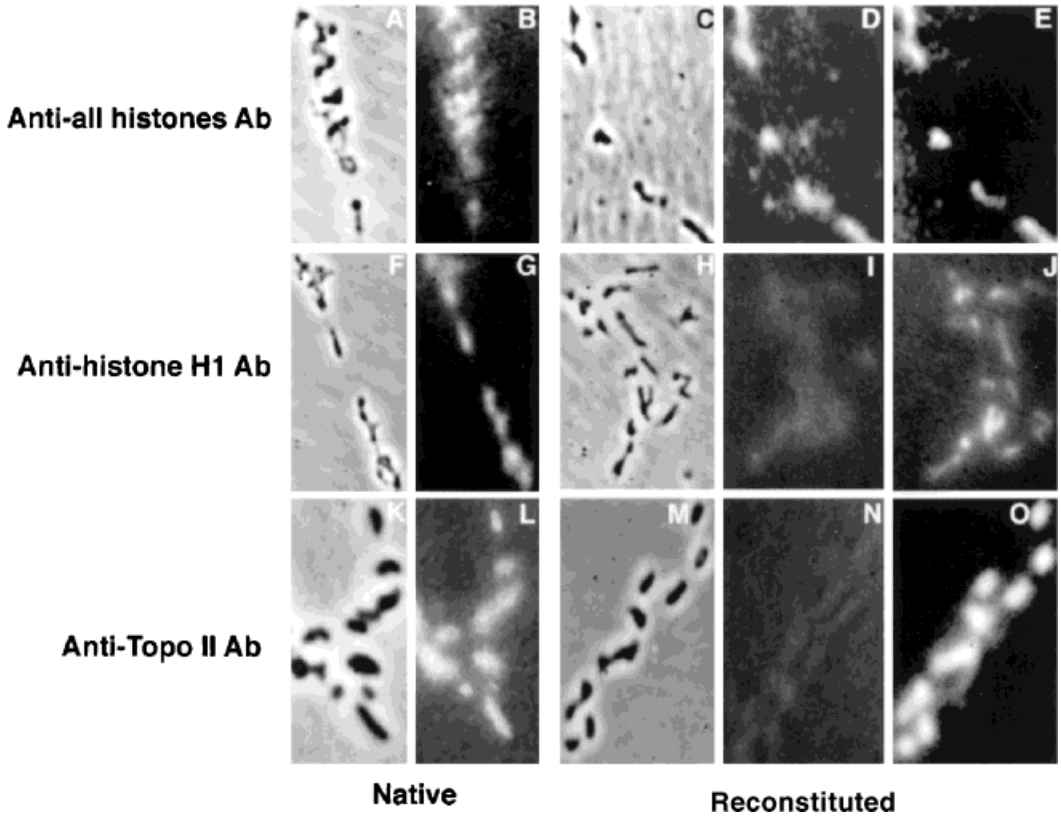

Fig. 6. Native pulled chromosomes (**A,B,F,G,K,L**) and proteolyzed chromosomes reconstituted by addition of exogenous Topo I (**C–E**), Topo II (**H–J**), or histone H1 (**M–O**). Isolated native chromosomes stained positively with antibodies against total histones (A, B), histone H1 (F, G), and Topo II (K, L); corresponding fluorescence views showing DAPI-stained reconstituted chromosomes are shown for comparison (E, J, O). While chromosomes reconstituted after proteolysis retained staining for total histones (C, D), most staining for endogenous Topo II (M, N) or histone H1 (H, I) was lost. Nevertheless, addition of exogenous Topo I, Topo II, or histone H1 was each independently sufficient to induce efficient chromosome reassembly in this system. (A–J: ×1,000; K–O: ×1,600.)

recondensation (Fig. 7E), which could not be significantly enhanced when a final dose of Topo II was added (Fig. 7F). Quantitation of these results using computerized image analysis (Fig. 7A–F, right) confirmed that Topo II induced chromosome reassembly in this model in a dose-dependent and saturable manner (Fig. 7G), which appeared to be based on stochiometric, rather than enzymatic effects.

To directly determine whether Topo II requires its catalytic activity to induce chromosome reassembly, we measured the catalytic strand-passing activity of Topo II in parallel by quantitating its ability to decatenate kinetoplast DNA. The purified enzyme preparation should not contain ATP, which is required for its catalytic activity. This was confirmed in the in vitro Topo II-specific decatenation assay, which revealed that the purified enzyme we used was catalytically active only in the presence of exogenous ATP (Fig. 8A). However, it was still possible that small amounts of ATP remained bound to the disassembled chromosomes, allowing the enzyme some level of strand passing activity in situ. To rule out this possibility, we added Topo II to proteolytically disassembled chromosomes in the presence of 200 μM of amsacrine (m-AMSA), a potent inhibitor of Topo II catalytic activity [Nelson et al., 1984]. Incubation of purified Topo II enzyme with m-AMSA for 10 min resulted in complete inhibition of its catalytic activity when analyzed in the in vitro enzyme assay (Fig. 8A). Nevertheless, Topo II inactivated in an identical manner with m-AMSA continued to effectively induce reassembly of proteolyzed chromosomes (Fig. 8B–D).

These results clearly demonstrate that the effects of Topo II on chromosome reassembly occur independently of its catalytic activity in this model system. However, the decatenation assay used to demonstrate that amsacrine blocks the catalytic activity of Topo II may require several rounds of strand passage to

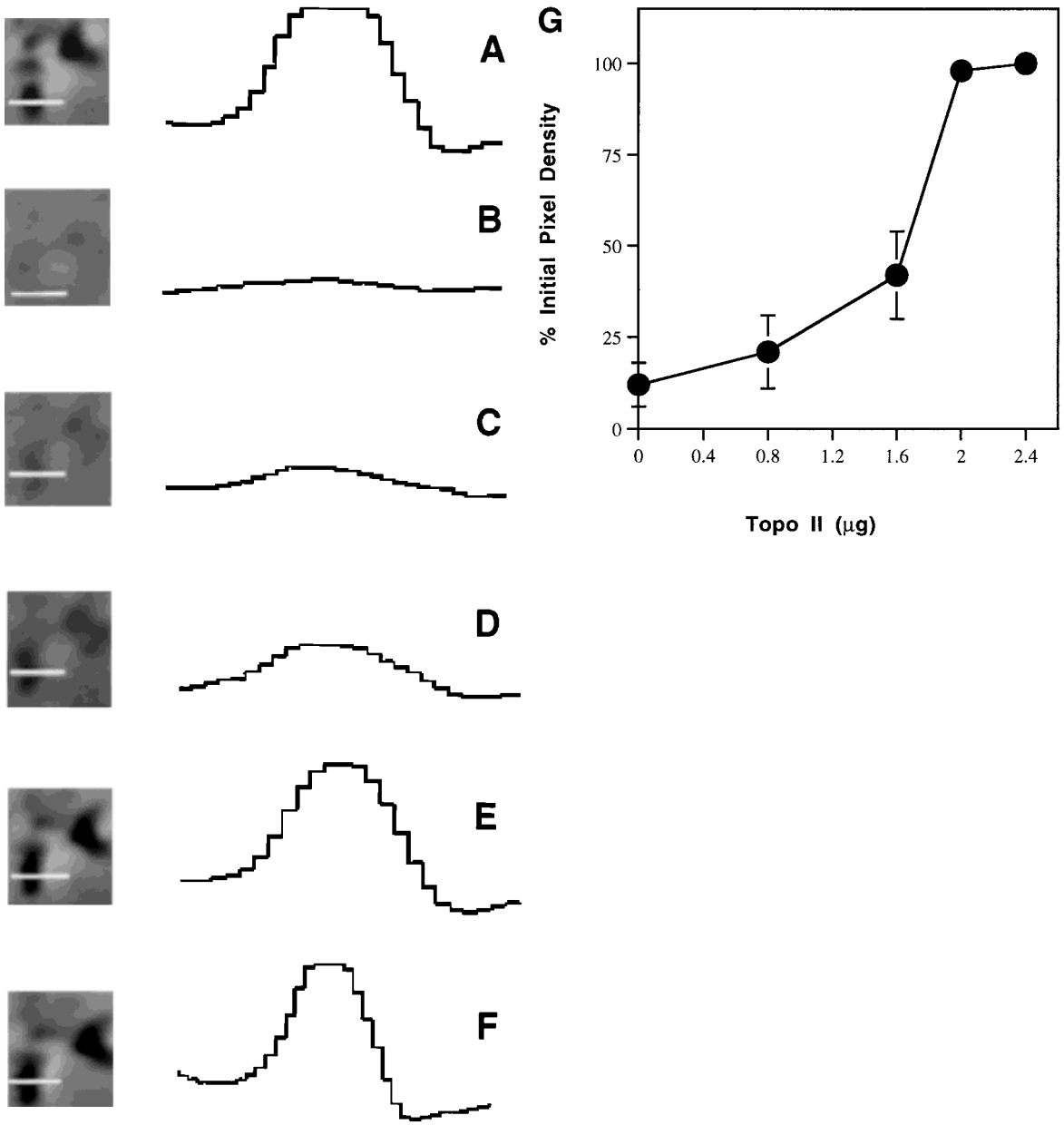

Fig. 7. Dose-dependent reassembly of protease-digested chromosomes by Topo II. **A:** Isolated chromosomes prior to protease exposure are shown at the left. A densitometric tracing of phase contrast pixel density measured along the horizontal white line drawn across the mid portion of the vertically-oriented chromosome at the left of this view is graphically depicted on the right. **B:** The same chromosome after disassembly by treatment with protease demonstrating nearly complete loss of morphology and phase contrast pixel density. **C:** The same disassembled chromosome after addition of Topo II (4 μl of 200 ng/μl) showing a slight increase in pixel density, but only minimal visible changes in chromosome form even after steady-state had been reached. **D:** The same view as in C after addition of another aliquot (4 μl) of Topo II and once again being allowed to reach a steady-state. A step-wise increase in chromosome condensation and pixel density resulted. **E:** Addition of another aliquot (2 μl) of Topo II resulted in nearly complete reconstitution of chromosome morphology and phase contrast pixel density. **F:** Addition of more Topo II (2 μl) did not produce any significant increase in chromosome condensation (×2,600). **G:** A graphical depiction of the results obtained in A–F using computerized densitometry, demonstrating that Topo II induced chromosome reassembly in a dose-dependent and saturable manner. These data represent the result of three independent experiments.

take place, before free DNA circles can be observed. Therefore, it is possible that under the conditions used, the enzyme is still able to carry out limited strand passage. In order to eliminate this possibility, we used a thermosensitive catalytic mutant of Topo II (top2-5), which displays catalytic activity at 25°C but not at 37°C. As previously reported [Jannatipour et al., 1993], we found that the catalytic activity of the top2-5 mutant was impaired at the restrictive

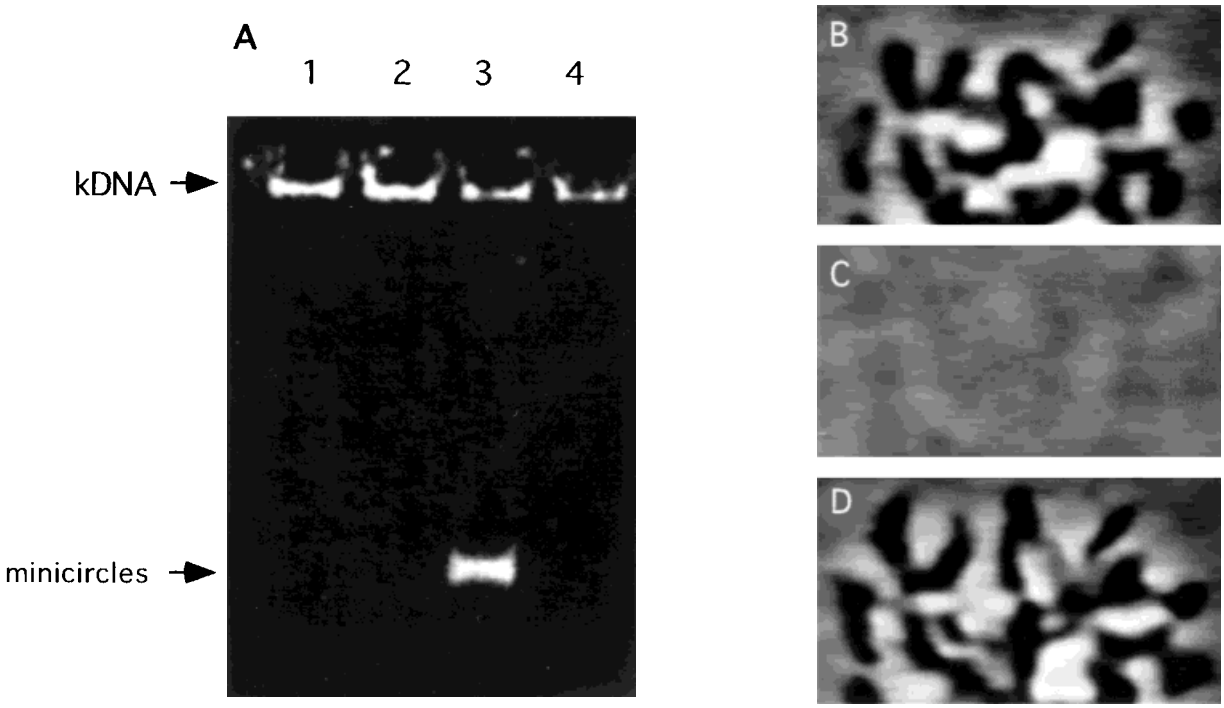

Fig. 8. Analysis of the relation between the DNA strand-passing activity of Topo II (A) and its ability to induce chromosome reassembly in vitro (B–D). **A:** Ethidium bromide–stained agarose gel showing results from an in vitro catalytic activity assay for Topo II carried out in the absence and presence of the enzyme inhibitor, m-AMSA. **Lane 1:** Kinetoplast DNA (kDNA) incubated in the absence of Topo II enzyme. **Lane 2:** Topo II (500 µg/ml) placed in the reaction mixture containing kDNA in the absence of ATP. **Lane 3:** The same reaction as in lane 2 carried out in the presence of 1 mM ATP. **Lane 4:** The same reaction as in lane 3 except that 200 µM m-AMSA was also included in the reaction buffer. Decatenation of kinetoplast DNA was carried on for 6 min, then the substrate (kDNA) and product (minicircles) of the reaction were resolved by electrophoresis on agarose gels and visualized by illumination with UV light. Positive Topo II catalytic activity was only observed in the presence of ATP and in the absence of m-AMSA in this study, as indicated by the production of an ethidium bromide–labelled minicircle band in the lower portion of the lane 3. **B:** Phase contrast micrograph of intact chromosomes isolated using microsurgery. **C:** The chromosomes shown in B after being disassembled by exposure to a mixture of trypsin and proteinase K. **D:** The same cluster of chromosomes showing reassembly induced by addition of Topo II (2 µg) in the presence of its inhibitor, m-AMSA (200 µM) (×3,200).

temperature (Fig. 9A), while its DNA binding activity remained unchanged (results not shown). Nevertheless, the top2-5 enzyme effectively induced changes in higher order chromosome architecture at both permissive (not shown) and non-permissive temperatures with similar kinetics. Furthermore, this was not due to random clumping of the DNA since several rounds of disassembly and reassembly could be induced at the restrictive temperature using this catalytically inactive Topo II, and every round resulted in reformation of chromosomes with similar shape and position (Fig. 9B–E).

Given that Topo II did not require its strand-passing activity to induce changes in chromosome structure, it remained unclear whether the Topo II we added bound to chromosomes in a manner that would permit it to physically interact with DNA. To explore this possibility, we treated Topo II-reassembled chromosomes with the non-hydrolyzable β,γ-amido analogue of ATP, AMP-PNP. This ATP analogue has been shown to inhibit Topo II catalytic activity by inducing stable dimerization of the ATP-binding domains of Topo II, thereby clamping this mechanoenzyme in its closed form around DNA during the last stage of the strand-passing cycle [Osheroff et al., 1983; Roca and Wang, 1992; Berger et al., 1996]. Freezing the DNA-Topo II complex in this manner makes it resistant to salt extraction [Osheroff, 1986; Roca and Wang, 1992]. For this reason, AMP-PNP can be used as a biological fixative to structurally stabilize whole chromosomes [Saitoh and Laemmli, 1994]. AMP-PNP did not produce any visible effect in our system when added to chromosomes that had been induced to reassemble by Topo II. However, this treatment effectively resulted in fixation of chromosome structure. For example, while Topo II-reconstituted chromosomes commonly disassemble when exposed to high salt (60–100 mM $MgCl_2$; Fig. 10A–C), AMP-

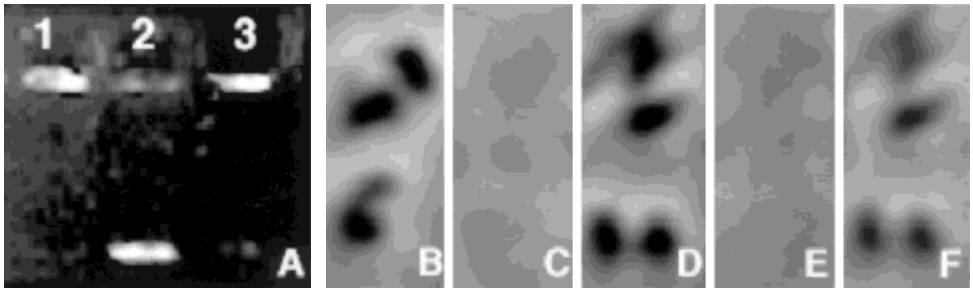

Fig. 9. Chromosome reconstitution with the temperature-sensitive Topo II catalytic mutant, top2-5. **A:** Agarose gel decatenation assay using substrate kDNA without enzyme (**lane 1**) or in the presence of the top2-5 mutant enzyme at 25°C (**lane 2**) or 37°C (**lane 3**). **B:** Native chromosomes. **C:** Same chromosomes digested by mixture of proteinase K and trypsin. **D:** Chromosomes resassembled with 1 µg of the catalytic top2-5 mutant at the restrictive temperature (37°C). **E:** Disassembly of the top2-5-reconstituted chromosomes shown in C after protease treatment. **F:** Subsequent reassembly of the same chromosomes after rinsing and addition of more (1 µg) top2-5 enzyme at 37°C (×2,600).

PNP-treated chromosomes failed to disassemble and remained almost entirely condensed, even when salt concentrations were raised as high as 300 mM (Fig. 10D–F).

DISCUSSION

It is known that Topo II is required for chromosome assembly [Uemura et al., 1987; Hirano and Mitchison, 1991; Adachi et al., 1991; Buchenau et al., 1993; Downes et al., 1994] and it is commonly assumed that the driving force behind Topo II's effects on chromosome form results from its enzymatic (DNA strand passing) activity and its associated effects on DNA topology [Uemura et al., 1987; Gorbsky, 1994; Downes et al., 1994; Giménez-Abián et al., 1995]. Topo II also has been suggested to play a structural role in chromosome organization based on its ability to bind to the nuclear matrix that forms the chromosome scaffold [Earnshaw et al., 1985; Gasser et al., 1986]; however, the relevance of these direct binding interactions for chromosome organization remains highly controversial [for review see Koshland and Strunnikov, 1996].

One reason that the function of Topo II in chromosome organization is not well understood is that this problem has eluded traditional experimental approaches. Specifically, it has not been possible to study chromosome structure and dynamics under defined conditions in vitro. In the present study, we took advantage of the recent development of a microsurgical procedure that permits removal of multiple intact chromosomes from living mammalian cells that can be disassembled and resassembled in vitro in the absence of fixation or chemical stabilization [Maniotis et al., 1997]. Using this approach, we showed that exogenous purified Topo II can incorporate into disassembled chromosomes and drive their reassembly into well-formed units with preexisting shape and position, even after more than 75% of endogenous Topo II and histone H1 were removed by proteolysis. Staining with DNA-binding dyes confirmed that proteolysis resulted in a profound loss of chromosome structure and DNA organization. This chromosome disassembly was nearly completely reversed by addition of Topo II, thus demonstrating the capacity of this enzyme to drive changes in higher order chromatin structure at the level of entire chromosomes. Furthermore, multiple rounds of disassembly and faithful reassembly could be induced, confirming that this response was specific and reversible.

Interestingly, two other nuclear proteins that contain stretches of cationic amino acid sequences (Topo I and histone H1) were also capable of fully reassembling proteolyzed chromosomes whereas many other molecules associated with the chromatin, such as Histone H2b, Histone H3, actin, and tubulin [Maniotis et al., 1997], as well as spermine and HIV-1 integrase (a protein that shares Topo II's DNA binding activity) were unable to reconstitute stable chromosome form. Topo I has been previously shown to play a role in the condensation of ribosomal DNA [Castano et al., 1996]. The critical role of histone H1 in the maintenance of higher order chromatin architecture is also well established [Renz et al., 1977; Thoma et al., 1979]. The similar effects of histone H1 and Topo II in this system could be explained by similarities in their lysine-rich, DNA binding domains [Lynn et al., 1986]. Although there are no apparent sequence homologies between Topo II and Topo I, they might have similarly structured cationic

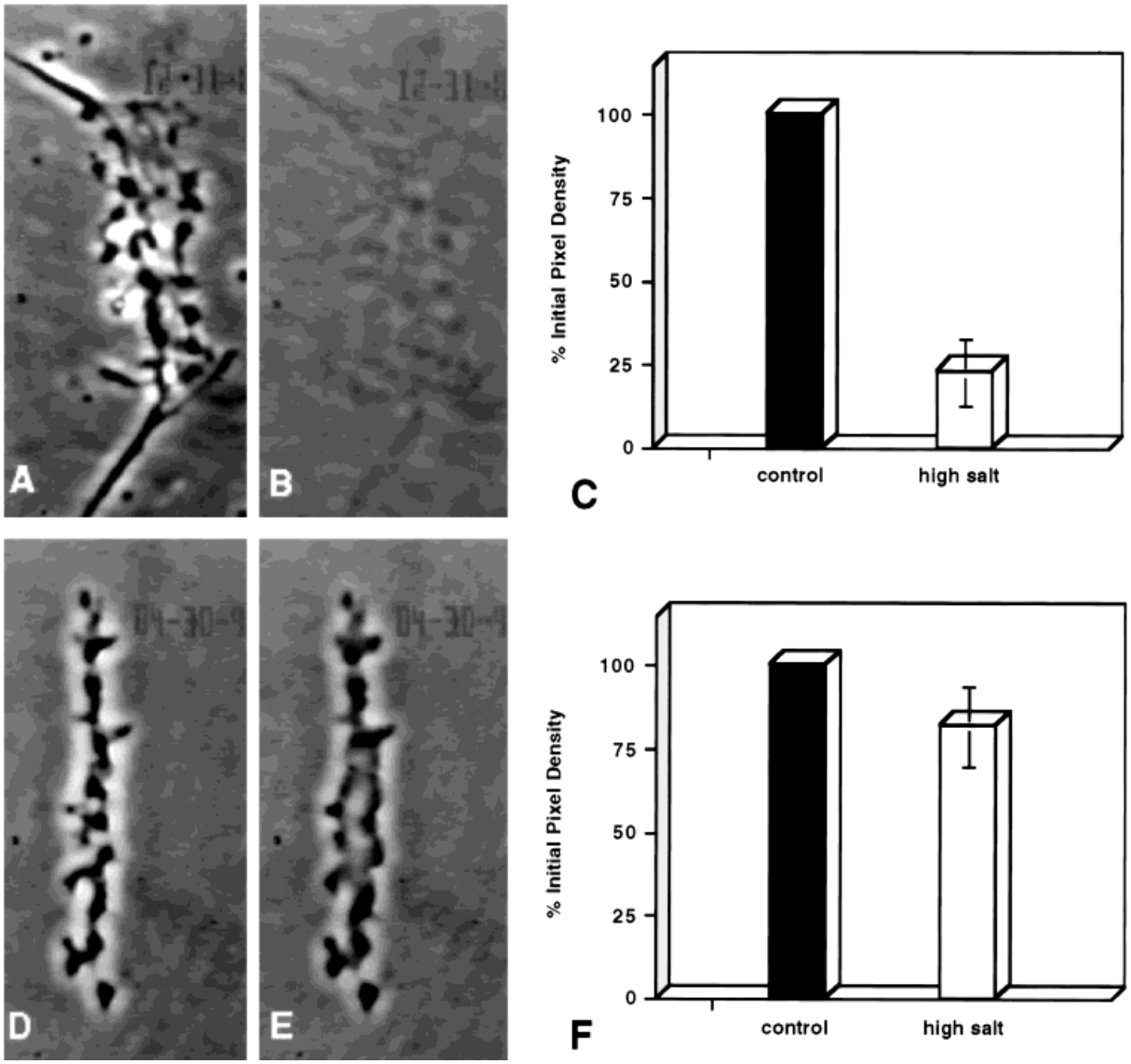

Fig. 10. Structural stabilization of Topo II-reconstituted chromosomes by AMP-PNP. **A:** A protease-treated chromosome chain after reassembly was induced by addition of Topo II. **B:** The same chain after addition of high salt (100 mM $MgCl_2$), showing that the reassembly process can be reversed. **C:** Densitometric quantitation of the salt-induced disassembly step; changes in average phase contrast pixel densities characteristic for a local ensemble of different chromosomes present in one constant region of the view were used to make these calculations. **D:** Another protease-treated chromosome chain after reassembly was induced by addition of Topo II. This chain was then treated with 2.5 mM AMP-PNP to lock Topo II dimers in a closed form around DNA. **E:** The same chain as in D after addition of 300 mM $MgCl_2$, demonstrating that AMP-PNP treatment results in structural stabilization of these chains. **F:** Densitometric quantitation of these results confirmed that pretreatment of Topo II-condensed chains with AMP-PNP resulted in almost complete suppression of the salt-induced disassembly response (×1,800).

domains. Accordingly, Histone H1, Topo II, and Topo I have the same binding preferences with regard to DNA topology [Zechiedrich and Osheroff, 1990; Caserta et al., 1994]. Thus, chromosome structure could be stabilized by electrostatic interactions between the polycationic regions of these proteins and DNA or other proteins, rather than by specific ligand-binding interactions or catalysis [Gronbech-Jensen et al., 1997].

The functional redundancies we observed in our in vitro model could exist in vivo since Topo II, Histone H1, and Topo I all remain associated with chromosomes during mitosis [Shen et al., 1995; Meyer et al., 1997]. Moreover, the possibility that Topo I and Topo II may be playing

structural roles in chromosomes is supported by the finding that the relative abundance of both enzymes increases 2–3-fold during mitosis while at the same time their specific activity drops [Meyer et al., 1997]. Functional redundancy is a well-known phenomenon in yeast where the disruption of gene coding for Topo I is not lethal as long as Topo II remains functional [Uemura and Yanagida, 1984]. Mutation of a SMC family member protein cut3 involved in chromosome condensation can also be complemented by overexpression of Topo I [Saka et al., 1994]. Such functional redundancies could account for enhanced structural stability of chromosomes in living cells.

In order to further analyze the molecular basis of the Topo II-DNA interaction within reconstituted chromosomes, we "locked" the conformation of the Topo II enzyme in a closed form clasped around DNA, by treating Topo II-reconstituted chromosomes with AMP-PNP. AMP-PNP exerts this effect during the last stage of the catalytic cycle, that is, after initial DNA binding and strand passing have taken place [Roca and Wang, 1992]. Treatment of Topo II-DNA complexes with AMP-PNP causes them to become resistant to salt extraction [Osheroff, 1986], apparently because the closed enzyme is unable to release the captured DNA strand [Osheroff, 1986; Roca and Wang, 1992; Berger et al., 1996]. Treatment of intact Topo II-reconstituted chromosomes with AMP-PNP similarly stabilized their form and made them highly salt-resistant. Chromosome-associated Topo II, therefore, appeared to retain its ability to physically interact with DNA in a physiological manner.

Our results demonstrate that Topo II molecules can act as building blocks in chromosome architecture, a possibility raised by others in the past [Earnshaw et al., 1985; Gasser et al., 1986; Adachi et al., 1991; Andreassen et al., 1997]. Furthermore, four lines of evidence suggest that the structural role of Topo II in the chromosome organization can be dissociated from its well-known catalytic activity. First, titration of disassembled chromatin with exogenous purified Topo II revealed that the reassembly response was controlled stoichiometrically, rather than catalytically. Specifically, repeated additions of suboptimal doses of Topo II induced step-like increases in chromosome condensation that proceeded at the same rate, but ended in a different final steady state, regardless of the time of exposure. Indeed, if this process were catalytic, addition of a low concentration of Topo II enzyme would alter the kinetics of reassembly and not its final product. This finding is in agreement with results from a past study, which analyzed the relationship between the amount of Topo II added and the degree of chromatin assembly in chicken erythrocyte nuclei incubated with Xenopus egg extracts [Adachi et al., 1991].

The second line of evidence was that Topo II-mediated changes in higher order chromosome architecture was achieved in the absence of exogenous ATP, a critical cofactor for its enzyme activity. The third was that purified Topo II effectively induced chromosome assembly even when the catalytic activity of the enzyme was inhibited by incubation with m-AMSA. These results correlate with a recent report claiming that the override of the G_2 block induced by another inhibitor of Topo II activity, VM-26, results in normal chromosome condensation [Andreassen et al., 1997]. The final and most convincing line of evidence came from our finding that a thermosensitive catalytic mutant of Topo II could effectively mediate chromosome reassembly at both the non-permissive and permissive temperatures. Furthermore, wild type and mutant Topo II drove chromosome reassembly with similar kinetics. Taken together, these data suggest that Topo II may control chromosome structure as a result of direct protein binding interactions, independently of its well-known ability to enzymatically modify DNA. The results raise the possibility that Topo II could have a non-catalytic role both during initial chromosome assembly and subsequent maintenance of condensed chromosome form in vivo, in addition to its recognized catalytic role in sister chromatid desentanglement. Understanding this new function of Topo II may lead to new approaches for anti-cancer therapy.

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