

Topoisomerase II Does Not Play a Scaffolding Role in the Organization of Mitotic Chromosomes Assembled in *Xenopus* Egg Extracts

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Abstract. We have investigated the role of topoisomerase II (topo II) in mitotic chromosome assembly and organization in vitro using *Xenopus* egg extracts. When sperm chromatin was incubated with mitotic extracts, the highly compact chromatin rapidly swelled and concomitantly underwent local condensation. Further incubation induced the formation of entangled thin chromatin fibers that eventually resolved into highly condensed individual chromosomes. This in vitro system made it possible to manipulate mitotic chromosomes in their assembly condition without any isolation or stabilization steps. Two complementary approaches, immunodepletion and antibody blocking, demonstrated that topo II activity is required for chromosome assembly and condensation. Once condensa-

tion was completed, however, blocking of topo II activity had little effect on the chromosome morphology. Immunofluorescent studies showed that topo II was uniformly distributed throughout the condensed chromosomes and was not restricted to the chromosomal axis. Surprisingly, all detectable topo II molecules were easily extracted from the chromosomes under mild conditions where the shape of chromosomes was well preserved. Our results show that topo II is essential for mitotic chromosome assembly, but does not play a scaffolding role in the structural maintenance of chromosomes assembled in vitro. We also present evidence that changes of DNA topology affect the distribution of topo II in mitotic chromosomes in our system.

THE structural organization of chromatin changes dramatically in the cell cycle. At the onset of mitosis, dispersed chromatin in the interphase nucleus is induced to condense, eventually producing mitotic chromosomes. Highly organized packing of chromatin into the condensed chromosomes and their proper interaction with the mitotic spindle are essential to ensure the fidelity of subsequent chromosome segregation. Although recent technical advances have made it possible to visualize spatial and temporal coordination of the dynamic chromatin behavior by real-time analysis in vivo (Hiraoka et al., 1989), little is known about the molecular mechanisms underlying dynamic changes of large scale chromosome structure during the progression of the cell cycle.

Amphibian egg extracts provide an ideal system to investigate chromosome assembly and its structural changes in vitro. Using a *Rana pipiens* egg extract, Lohka and Masui (1983) originally developed an in vitro system in which sperm chromatin was induced to form interphase nuclei and then converted into condensed chromosomes. More recently, it has been demonstrated that, in cell cycle-specific *Xenopus* egg extracts, naked DNA is capable of acting as a template for assembly of both interphase nuclei (Newmeyer et al., 1986; Blow and Laskey, 1986; Newport, 1987) and mitotic chromosomes (Hirano and Mitchison, 1991).

Topoisomerase II (topo II)¹ is an enzyme that regulates the topological structures of DNA by transient breakage and rejoining of double-stranded DNA. Genetic studies in yeasts have shown that topo II is required for condensation as well as segregation of mitotic chromosomes (Uemura et al., 1987). Involvement of topo II in condensation has also been suggested by in vitro experiments using *Xenopus* egg extracts: addition of a topo II inhibitor VM-26 blocked condensation of both rat liver nuclei (Newport and Spann, 1987) and chromosome-like structures assembled around naked DNA (Hirano and Mitchison, 1991). One problem with these inhibitor experiments is that it is not clear if the inhibition of condensation was due to a specific inhibition of topo II activity or a nonspecific effect of protein-linked DNA gaps induced by the drug treatment (Chen et al., 1984). Recently the requirement for topo II in chromosome condensation has been demonstrated in vitro by specific immunodepletion using somatic cell and *Xenopus* egg extracts (Wood and Earnshaw, 1990; Adachi et al., 1991). These studies used interphase nuclei with variable levels of endogenous topo II as substrates and showed that chromosome condensation is closely correlated with the level of topo II present in the reac-

1. *Abbreviations used in this paper:* EtBr, ethidium bromide; topo II, topoisomerase II.

tion. Specifically topo II-depleted extracts were able to induce HeLa nuclei to condense, but failed to produce mitotic chromosomes from chicken erythrocyte nuclei which have a low level of endogenous topo II.

In addition to its catalytic activity, topo II may also play a structural role in the maintenance of mitotic chromosomes since it has been identified as a major component of the chromosome scaffold (Earnshaw et al., 1985; Gasser et al., 1986). The chromosome scaffold was originally characterized as a residual framework of metaphase chromosomes after histone extraction (Paulson and Laemmli, 1977) and is thought to anchor the chromatin loop domains (reviewed by Gasser et al., 1989). DNA sequences that preferentially bind to the chromosome scaffold (called scaffold-associated region [SAR]) was found to contain a cluster of topo II cleavage consensus. Taken together, it has been proposed that topo II plays a scaffolding role in the organization of the chromatin loops via a direct interaction with SAR (Gasser and Laemmli, 1987).

The existence of the scaffold structure in intact mitotic chromosomes has, however, remained controversial. The original harsh conditions for scaffold preparation have been suspected to potentially induce formation of an artifactual aggregation of proteins (Okada and Comings, 1980; Hadlaczký et al., 1981; Paulson, 1989). Recent analyses using mAbs raised against the chromosome scaffold fraction revealed that the fraction consists not only of chromosomal proteins, but also of other proteins that normally bind to the mitotic spindle (reviewed by Earnshaw, 1991; Compton et al., 1991, 1992). Substantial changes of antigen distribution may be induced in drug-arrested mitotic cells in vivo before chromosome isolation. Thus, alternative experimental procedures will be required for dissecting mitotic chromosomes in a condition where they are actually assembled.

In this report, we used sperm chromatin, which was found to lack detectable amounts of endogenous topo II, to investigate the specific role of topo II in mitotic chromosome assembly and organization in vitro. Using *Xenopus* egg extracts in which the level of active topo II molecules can be immunochemically manipulated, we present evidence that topo II is required for mitotic chromosome assembly and condensation, but does not play a scaffolding role in the structural maintenance of chromosomes assembled in this system. We also show that this in vitro system is useful for manipulating mitotic chromosomes in their assembly condition without any isolation or stabilization steps.

Materials and Methods

Preparation of Mitotic Extracts

Mitotic extracts were prepared from eggs of *Xenopus laevis* as described previously (Newport and Spann, 1987; Murray et al., 1989; Hirano and Mitchison, 1991) with minor modifications. In brief, mitotic crude extracts were prepared by crushing unfertilized eggs in EB (80 mM β -glycerophosphate, pH 7.3, 15 mM MgCl₂, 20 mM EGTA and 1 mM DTT, with 10 μ g/ml leupeptin, chymostatin, and pepstatin). The crude extracts were further fractionated by ultracentrifugation at 50,000 rpm for 2 h at 4°C (TLS-55 rotor; Beckman Instruments, Inc., Palo Alto, CA). The soluble fractions were carefully removed using a 20-gauge needle, and recentrifuged at 50,000 rpm for 30 min to remove residual membranes. 50- μ l aliquots were frozen in liquid nitrogen and stored at -80°C (high-speed supernatants). We found that in some batches of extracts slight dilution of extracts (up to 2-fold) produced better results than those obtained from undiluted extracts. Twofold

dilution of extracts did not affect the efficiency of nucleosome assembly on a closed circular plasmid DNA as judged by a supercoiling assay.

Preparation of Sperm Chromatin

Demembrated *Xenopus* sperm chromatin was prepared by the method described by Gurdon (1976), and stored in 30% glycerol at -80°C.

Cloning of cDNAs Encoding *Xenopus* Topo II

To prepare specific antisera against the *Xenopus* topo II protein, we isolated cDNAs encoding *Xenopus* topo II and bacterially expressed fusion proteins to be used as antigens. We selected two amino acid stretches of topo II that are completely conserved among four eukaryotic species (Giaever et al., 1986; Uemura et al., 1986; Tsai-Pflugfelder et al., 1988; Wyckoff et al., 1989) and designed a set of polymerase chain reaction (PCR) primers. Primer 1 encodes the amino acid sequence LAQNFVG (amino acid 770-776 in human topo II) on the coding strand, and primer 2 encodes the sequence NGAEGIG (amino acid 850-856) on the noncoding strand. The nucleotide sequence of primer 1 is 5'GATGGATCC(TC)T(ATGC)GC(ATGC)CA(AG)AA(TC)TT(TC)GT(ATGC)GG3' (1,024-fold degenerate). The sequence for primer 2 is 5' GACCTGCAGCC(ATG)AT(ATGC)CC(TC)TC(ATGC)GC(ATGC)CC(AG)TT3' (768-fold degenerate). All nucleotides in parentheses were included at that position.

Poly(A)⁺ RNA was isolated from *Xenopus* eggs using FastTrack mRNA isolation kit (Invitrogen, San Diego, CA) according to manufacturer's instruction. cDNA produced from the RNA with oligo(dT) priming was used as a template for PCRs. A reaction amplified a single band with an expected length of 270 bp. A *Xenopus* embryo Agt10 cDNA library (kindly provided by E. Amaya and M. W. Kirschner, University of California, San Francisco, CA) was screened using this PCR fragment as a probe and two classes of cDNA were obtained. The first class of cDNA (designated XT-1) was ~6.5 kb long and contained the entire length of topo II coding region. The second class (designated XT-2) was ~3 kb long and was missing the NH₂-terminal half of coding region. Alignment of these two sequences and those of human topo II isotypes (Chung et al., 1989) revealed that XT-1 and XT-2 appear to encode the α (170-kD form) and β (180-kD form) type of topo II, respectively. We also found that XT-1 (topo II α) was highly expressed in proliferating cells such as eggs and embryos, whereas XT-2 (topo II β) was predominantly expressed in nonproliferating G₀ cells such as liver. This is in good agreement with the differential expression pattern of topo II isotypes reported in mammalian cells (Woessner et al., 1991) and supports the conclusion described above.

Preparation of Anti-topo II Antisera

A fusion protein containing a COOH-terminal domain of topo II α was expressed in *E. coli* using T7 RNA polymerase-dependent expression system (Studier et al., 1990). A 3.8-kb HindIII fragment of the *Xenopus* topo II α cDNA was subcloned into a cloning vector pGEM7Zf(+) (Promega Corp., Madison, WI). The resulting plasmid pXT115 was digested with BglII and BamHI, and a 3.0-kb BglII-BamHI fragment was ligated into an expression vector pET11c (Studier et al., 1990) to generate pXT1135, which can express an in-frame fusion of T7 gene 10 product (11 amino acids) and a COOH-terminal domain (~800 amino acids) of topo II α . The plasmid was introduced into an *E. coli* strain BL21(DE3), which contained the T7 RNA polymerase gene under the control of *lac* UV5 promoter. Expression of the fusion protein was induced for 3 h by the addition of 1 mM isopropyl- β -thiogalactopyranoside (IPTG) and inclusion bodies were isolated as described by Sambrook et al. (1989). The insoluble materials were resolved in preparative SDS-polyacrylamide gels, and after brief staining with Coomassie blue, fusion protein bands were cut out. The polypeptides were electroeluted in 20 mM sodium phosphate, pH 7.5, containing 0.1% SDS, dialyzed against TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and used as antigens. Immunizations and bleeds were carried out by the Berkeley Antibody Company (Richmond, CA). Two antisera from different rabbits recognizing a common polypeptide of 180 kD, which corresponds well to the reported size of *Xenopus* egg topo II (Luke and Bogenhagen, 1989). One of them recognizing a single band of 180 kD on blots was used for depletion experiments. Affinity purification of anti-topo II antibodies was performed as follows. The gel-purified fusion protein was dialyzed against 0.1 M MOPS, pH 7.5, and was coupled to Affigel 10 agarose (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. An anti-topo II antiserum was passed over the fusion protein column three times, and the column was washed initially with TBS and then with 20 mM Tris-HCl, pH 7.5/0.5 M NaCl. Anti-topo II IgG was eluted with 0.2 M glycine-

HCl, pH 2.5. The peak fractions were pooled, dialyzed against TBS, and stored at 4°C. The affinity-purified anti-topo II IgG was found to inhibit decatenation activity of topo II *in vitro*.

Immunodepletion of Topo II

Protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO) was washed five times with TBS, mixed with an equal volume of either rabbit antiserum raised against the COOH-terminal domain of the *Xenopus* topo II α or preimmune serum collected from the same rabbit, and incubated at 4°C for 1–2 h. The Sepharose was washed three times with TBS and twice with EB. After the final wash, residual buffer was removed as completely as possible using a 23-gauge needle. Undiluted extract was added to an equal volume of the antibody-loaded Protein A-Sepharose and incubated on ice for 1 h with occasional agitation. The treated extract was then separated from the Sepharose by centrifugation, and used as the depleted extract. To confirm the specificity of depletion, topo II–depleted extracts were also prepared using affinity-purified anti-topo II IgG (see above) and used in some experiments.

Assays for Topo II Activity and Nucleosome Assembly

Topo II activity was assayed for the ability to decatenate kinetoplast DNA (Marini et al., 1980). Kinetoplast DNA (TopoGEN, Inc., Columbus, OH) was added to twofold diluted extracts at a final concentration of 10 $\mu\text{g}/\text{ml}$ and incubated at 23°C. At appropriate intervals, the reactions were terminated by adding 20 μl of stop solution (20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% SDS, and 500 $\mu\text{g}/\text{ml}$ Proteinase K). After incubation at 55°C for 60 min, the reaction mixture was deproteinized with phenol and the DNA was precipitated with ethanol. After centrifugation the pellet DNA was dissolved in 12 μl of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and electrophoresed on a 0.8% agarose gel. Nucleosome assembly was examined by a supercoiling assay as described previously (Hirano and Mitchison, 1991).

Assays for Mitotic Chromosome Assembly and Condensation

Standard reactions for mitotic chromosome assembly contained 20 μl of mitotic extract (high-speed supernatant), 1 μl of sperm chromatin (final concentration of 2×10^5 nuclei/ml), and an ATP-regenerating system (final concentration of 1 mM ATP, 10 mM creatine phosphate, and 50 $\mu\text{g}/\text{ml}$ creatine kinase). Reactions were incubated at room temperature (23°C) for up to 3 h. To monitor structural changes of sperm chromatin, aliquots were removed at different time points, mixed with an equal vol of fixative (50% [wt/vol] glycerol, 3.7% formaldehyde, and 1 $\mu\text{g}/\text{ml}$ Hoechst 33258 in MMR), and observed by fluorescent microscopy (Photoscope III; Carl Zeiss, Inc., Thornwood, NY). MMR consisted of 100 mM NaCl, 2 mM KCl, 1 mM MgSO_4 , 2 mM CaCl_2 , 0.1 mM EGTA, and 5 mM HEPES, pH 7.8.

Topo II Readdition and Antibody Blocking

Highly purified *Drosophila* topo II was a generous gift of J. Swedlow (University of California, San Francisco, CA). This enzyme was purified from *Drosophila* embryos by the method of Shelton et al. (1983). On SDS-PAGE, it consists of a predominant band of 170 kD and two minor bands (135 and 155 kD) that are most likely to represent degradation products of the major band. Based on Luke and Bogenhagen (1989) and our quantitative immunoblot analyses, we estimate that topo II concentration in our high-speed supernatants is $\sim 2 \mu\text{g}/\text{ml}$. We found that the addition of *Drosophila* topo II at the physiological concentration was sufficient to restore condensation activity in topo II–depleted extracts (at a final concentration of 1 $\mu\text{g}/\text{ml}$ topo II in twofold diluted extracts; see Fig. 3). For antibody blocking experiments, 1/10 vol of TBS containing different concentrations of affinity-purified anti-topo II IgG were added. As negative controls, the same concentrations of normal rabbit IgG (Sigma Chemical Co.) were used. VM-26 (4'-demethylepipodophyllotoxin thenylidene- β -D-glucoside; a generous gift of Bristol-Meyers, Syracuse, NY) was dissolved in DMSO as a stock solution of 10 mM and diluted with EB immediately before use. We found that the final DMSO concentration of up to 1.0% did not affect mitotic chromosome assembly and condensation.

Immunofluorescence and Immunoblotting

Immunofluorescent staining was performed as described previously (Hirano and Mitchison, 1991). For topo II staining, affinity-purified anti-

topo II IgG was used at a concentration of 1 $\mu\text{g}/\text{ml}$. MPM-2 (generously provided by P. N. Rao, The University of Texas, M.D. Anderson Cancer Center, Houston, TX; Davis et al., 1983) was used at a dilution of 1:500. Immunoblotting was performed as described by Harlow and Lane (1988) using ECL (enhanced chemiluminescence) detection system (Amersham Corp., Arlington Heights, IL).

Extraction of Mitotic Chromosomes in Assembly Mixtures

Mitotic chromosomes were assembled in the standard assembly mixtures (prepared in EB) for 2 h and then mixed with an equal vol of EB containing increasing concentrations of NaCl. The treatment buffer contained EB plus 2 \times concentrated NaCl so that buffer composition of the final mixture was adjusted to EB plus 1 \times NaCl. After a 10-min incubation at room temperature, the chromosomes were fixed and processed for immunofluorescence. Alternatively, assembly mixtures were layered on EB containing 30% sucrose and spun at 12,000 rpm for 15 min. After washing interface five times with EB, chromosomes recovered in the pellet were suspended in a SDS-sample buffer and analyzed by immunoblotting. Dextran sulfate extraction was performed in the same way. In some experiments, chromosomes were pretreated with metal ions before extraction.

Results

Structural Rearrangements of Sperm Chromatin in Mitotic Extracts

Lohka and Masui (1984) originally found that an amphibian egg extract prepared in the presence of EGTA was able to directly convert sperm chromatin into a condensed state. We have investigated in detail intermediate stages of mitotic chromosome assembly and condensation using *Xenopus* egg mitotic extracts. Demembrated sperm chromatin exhibits a highly compact, snake-like shape (Fig. 1 a). Upon incubation with mitotic extracts, this compact chromatin rapidly swelled, increasing more than threefold in both length and width, and concomitantly underwent local condensation (Fig. 1 b). After 60 min, thin chromatin fibers (0.2–0.4 μm in diameter) became clearly visible in a chromatin mass (Fig. 1 c). The fibers were entangled with each other, making it difficult to trace the entire length of a single individual fiber. After 90–120 min, condensation proceeded and thicker, rod-shaped chromosome structures ($\sim 0.8 \mu\text{m}$ in diameter) were observed (Fig. 1 d). At this time point, individual chromosomes were clearly resolved, which were clustered in a mass (Fig. 1 d) or dispersed into the extract (Fig. 1 e). The number of chromosomes observed in each mass corresponded roughly to that of the haploid genome of *Xenopus laevis* ($n = 18$; Tymowska and Kobel, 1972). Not surprisingly, we did not find sister chromatids in the chromosome because sperm chromatin was directly induced to condense in the absence of DNA replication in this assay. The condensation state of the final products was similar to that of mitotic chromosomes that were induced to condense after DNA replication was completed in interphase nuclei (data not shown, see also Sawin and Mitchison, 1991). The structural changes of sperm chromatin proceeded in a highly synchronous manner: at most time points all chromatin structures were in a similar condensation state. We classified the discrete stages in chromatin rearrangement as follows: (a) swelling of highly compact sperm chromatin; (b) local condensation; (c) formation of entangled thin fibers; and (d) resolution of highly condensed chromosomes. Although the first two stages simultaneously occur in mitotic extracts, we were able to distinguish them experimentally in terms of topo II requirements as shown below.

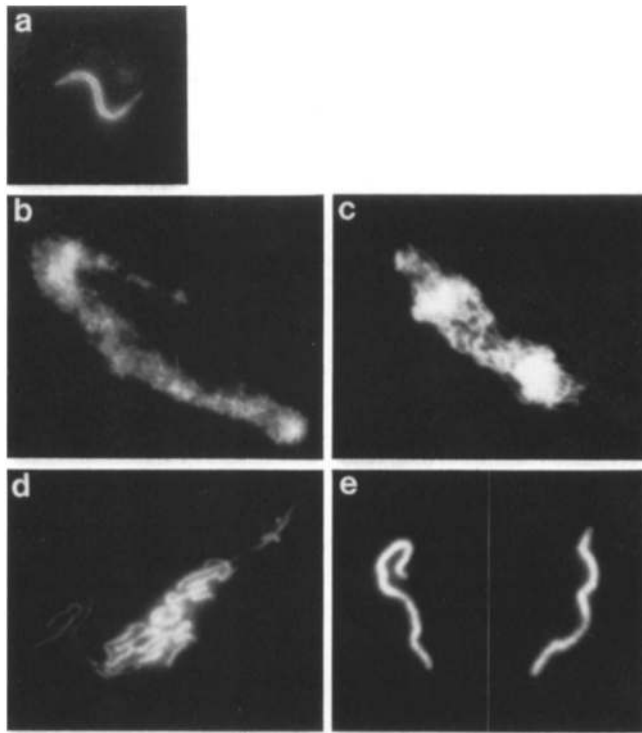


Figure 1. Structural rearrangements of sperm chromatin in mitotic extracts. Demembrated sperm chromatin exhibits a highly compact snake-like shape (a). The chromatin was incubated with a mitotic extract at 23°C. After 30-min (b), 1-h (c), and 2-h (d and e) incubations, condensation intermediates were fixed and stained with Hoechst 33258. Individual chromosomes were often found dissociated from a mass of condensed chromosomes (e). Bars: (a, b, c, and d) 10 μ m; (e) 5 μ m.

Immunodepletion of Topo II from Extracts

We wished to ask whether topo II plays an essential role in chromosome assembly and condensation in this assay. The most direct approach to address this question is either to deplete topo II molecules from extracts or to block topo II activity by the addition of anti-topo II antibodies. For this purpose, we cloned the *Xenopus* topo II cDNAs and prepared specific anti-topo II antisera against bacterially expressed recombinant proteins (see Materials and Methods). In the course of this study, we found that *Xenopus* has two isoforms of topo II, α and β , as recently found in mammalian cells (Drake et al., 1989; Chung et al., 1989). Northern and Western blot analyses using isotype-specific probes revealed that only the α type of topo II is expressed in *Xenopus* eggs although the β type was predominantly detected in *Xenopus* liver nuclei (our own unpublished results). Consistent with this result, when topo II α was depleted from egg extracts, no residual topo II activity was detected (see below). Taken together, we concluded that the expression of topo II β is extremely low (<1% of α) or absent in *Xenopus* eggs. On an immunoblot against *Xenopus* egg extracts, anti-topo II α recognized a single band of 180 kD (Fig. 2 a), that corresponded well to that of *Xenopus* topo II biochemically characterized in oocytes and eggs (Benedetti et al., 1983; Luke and Bogenhagen, 1989). Sperm chromatin was found to lack detectable levels of both isoforms of endogenous topo II as judged by

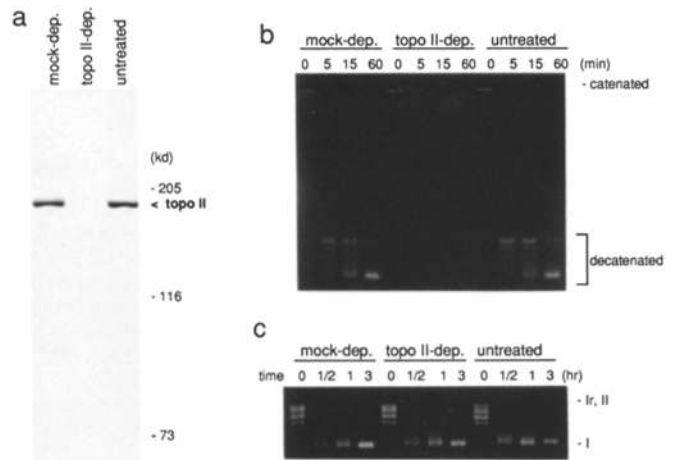


Figure 2. Immunodepletion of topo II. (a) Immunodepletion of topo II polypeptides from extracts. A mitotic extract was treated with an anti-topo II antiserum or with a preimmune serum to prepare topo II-depleted and mock-depleted extracts, respectively. Equal volumes of these extracts and an untreated extract were subjected to 7.5% SDS-PAGE, blotted on a nitrocellulose paper, and probed with the anti-topo II antibody. (b) Immunodepletion of topo II activity. Kinetoplast DNA was incubated with the mock-depleted, topo II-depleted, and untreated extracts at 23°C. After 5-, 15-, and 60-min incubations, aliquots were taken and the reactions were terminated. The DNA was deproteinized and analyzed on a 0.8% agarose gel. In mock-depleted and untreated extracts, catenated DNA (catenated) was rapidly decatenated to produce relaxed minicircles and then converted into supercoiled ones as a result of nucleosome assembly (decatenated). (c) Nucleosome assembly in the topo II-depleted extract. Nucleosome assembly activity was measured as supercoiling of relaxed circular plasmid DNA. Relaxed plasmid DNA was added to the mock-depleted, topo II-depleted, and untreated extracts at a final concentration of 10 μ g/ml and incubated at 23°C. After 0, 1/2, 1, and 3 h, aliquots were taken and the reactions were terminated. The DNA was analyzed as in b. I, fully supercoiled DNA; II, relaxed circular DNA; III, nicked circular DNA.

immunoblotting and immunofluorescent staining (data not shown). In this paper we investigated the role of topo II α that is recruited from egg extracts to sperm chromatin. For simplicity we use the word "topo II" to refer to topo II α throughout the text unless otherwise indicated.

We used the anti-topo II antisera to deplete topo II polypeptides from egg extracts. By treating a mitotic extract with protein A-Sepharose beads that had been preloaded with an anti-topo II antiserum, >95% of topo II polypeptides were depleted from the extract as judged by immunoblotting (Fig. 2 a). As a negative control, a preimmune serum collected from the same rabbit was used to prepare a mock-depleted extract. Topo II activity in the extracts was assayed for the ability to decatenate kinetoplast DNA (Fig. 2 b). In untreated and mock-depleted extracts, kinetoplast DNA was rapidly decatenated and converted into minicircles. In contrast, in topo II-depleted extracts, no decatenation activity was detected even after longer incubation. We also found that topo II depletion has no effect on the efficiency of nucleosome assembly on a closed circular plasmid DNA (Fig. 2 c). This is consistent with the previous reports that the torsional stress induced on a circular plasmid DNA during nucleosome assembly in vitro is mostly removed by topoisom-

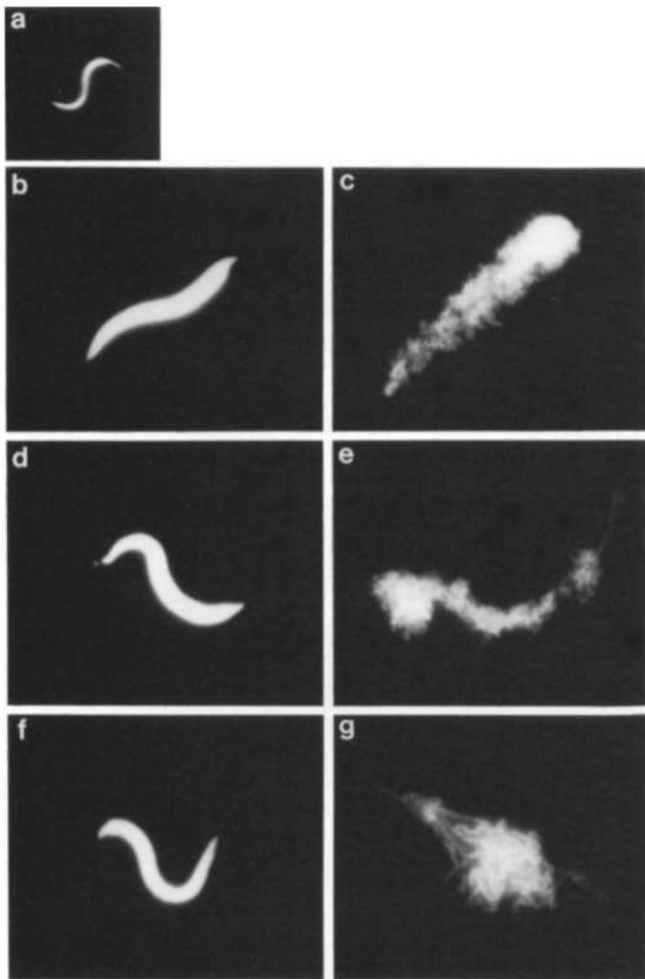


Figure 3. Topo II is required for mitotic chromosome assembly and condensation. Highly compact sperm chromatin (a) was mixed with a topo II-depleted mitotic extract (b, d, and f), and the same extract complemented with highly purified *Drosophila* topo II at a final concentration of 1 $\mu\text{g/ml}$ (c, e, and g). This concentration of topo II was comparable to the physiological level (see Materials and Methods). After 30-min (b and c), 1-h (d and e), and 2-h (f and g) incubations, chromatin structures were fixed, and stained with Hoechst 33258. Bar, 10 μm .

erase I (topo I) rather than topo II (Almouzni and Méchali, 1988).

Topo II Is Required for Mitotic Chromosome Assembly and Condensation

The requirement for topo II in chromosome assembly and condensation was examined using the topo II-depleted mitotic extracts. In the absence of topo II, highly compact sperm chromatin (Fig. 3 a) rapidly swelled but the subsequent structural changes were completely blocked (Fig. 3, b, d, and f). The swollen chromatin was uniformly stained with Hoechst, and showed no indication of local condensation or formation of thin chromatin fibers even after longer incubation (at least 5 h). We obtained identical results using extracts depleted with another anti-topo II antiserum collected from a different rabbit or with affinity-purified anti-topo II IgG (see Materials and Methods). In mock-depleted extracts prepared using the preimmune serum, sperm chromatin

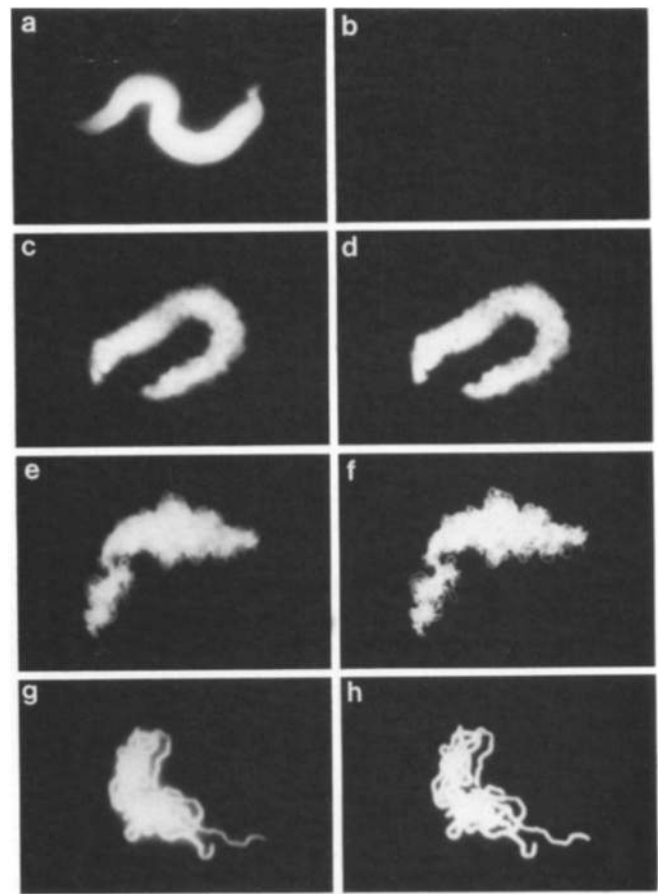


Figure 4. Localization of topo II on condensation intermediates. Sperm chromatin was incubated with topo II-depleted (a and b) and mock-depleted mitotic extracts (c-h). After 15 min (c and d), 1 h (e and f), and 2 h (a, b, g, and h), chromatin structures were fixed, and stained with Hoechst 33258 (a, c, e, and g) and affinity-purified anti-topo II (b, d, f, and h). Bar, 10 μm .

showed the identical structural changes to those observed in untreated extracts (data not shown).

Another control for the specificity of depletion is to add back purified topo II molecules. The addition of highly purified *Drosophila* topo II into the topo II-depleted extracts at a physiological concentration (see Materials and Methods) restored the condensation activity (Fig. 3, c, e, and g). The time course of structural changes in the complemented extract was very similar to those observed in untreated extracts. After a 2-h incubation highly condensed individual chromosomes were formed. Final condensation state of these chromosomes was indistinguishable from that produced in control extracts (Fig. 1 d and Fig. 3 g). These results show that topo II is not required for the swelling step but is essential for the subsequent stages of chromosome assembly and condensation.

Localization of Topo II and MPM-2 Antigens on Mitotic Chromosomes

We next investigated the localization of topo II molecules that are recruited from extracts onto chromatin structures by immunofluorescence. In topo II-depleted mitotic extracts, anti-topo II did not stain decondensed snake-shaped chroma-

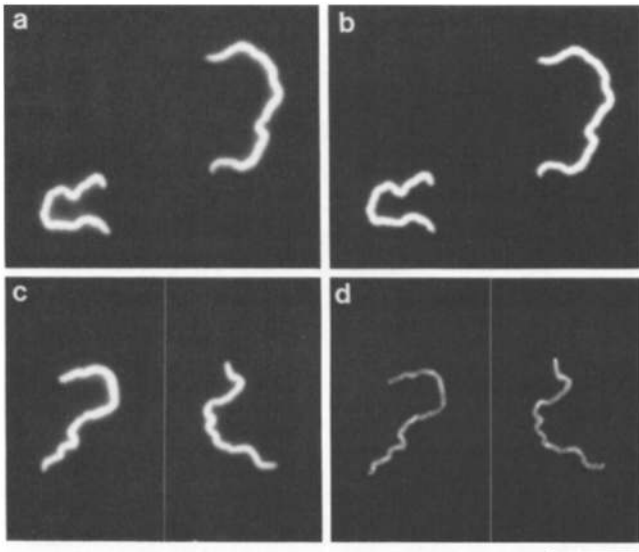


Figure 5. Localization of topo II and MPM-2 antigens on highly condensed chromosomes. Sperm chromatin was incubated with mock-depleted mitotic extracts for 2.5 h. Assembled chromosomes were fixed, and stained with Hoechst 33258 (a and c), affinity-purified anti-topo II (b), and a mAb MPM-2 (d). Bar, 10 μ m.

tin structures as expected (Fig. 4 b). In contrast, chromatin structures incubated with mock-depleted extracts were strongly stained with anti-topo II. Topo II molecules appeared on the chromatin from early stages of condensation (Fig. 4 d), and were uniformly distributed on the entire length of entangled thin chromatin fibers (Fig. 4 f) and of highly condensed chromosomes (Fig. 4 h). The staining patterns of these structures with anti-topo II were very similar to those with a DNA binding dye Hoechst (Fig. 4, c, e, and g). In fully resolved chromosomes, topo II was distributed throughout the whole structures and was not restricted to the chromosomal axis (Fig. 5 b). Exogenously added *Drosophila* topo II in complementation experiments was also found to show similar distribution (data not shown).

We have previously shown that a mAb MPM-2, which recognizes a class of mitosis-specific phosphoproteins (Davis et al., 1983), stains both a "condensation core" of mitotic chromosome-like structures assembled around naked DNA and a central axis of mitotic chromosomes isolated from CHO cells (Hirano and Mitchison, 1991). The strong correlation between the assembly of this substructure and the condensed state of the chromosomes suggested that the MPM-2 antigens may play an essential role in mitotic chromosome assembly and condensation. Consistent with the previous observation, we found that MPM-2 antigens are concentrated on axial region of mitotic chromosomes assembled from sperm chromatin in vitro (Fig. 5 d). The staining pattern was clearly different from the distribution of topo II (Fig. 5 b).

Topo II Activity Is Not Required for Structural Maintenance of Mitotic Chromosomes

We next asked if topo II is involved in the structural maintenance of mitotic chromosomes assembled in this system. It has been proposed that topo II plays a "loop-fastener" role in the organization of the chromatin loop domains (Gasser

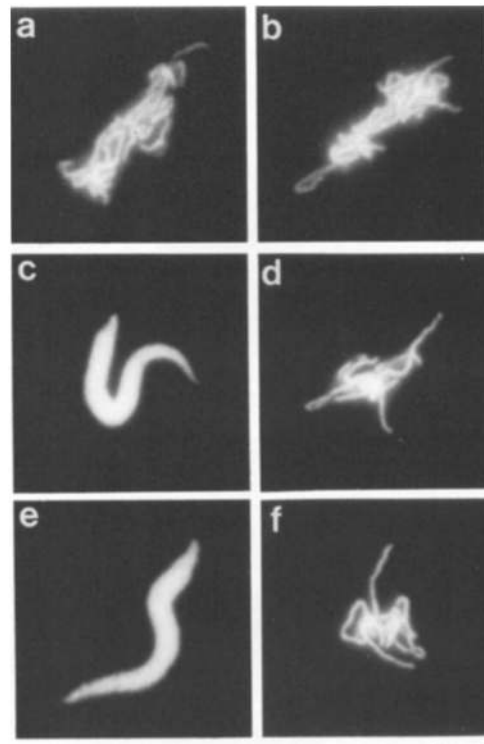


Figure 6. Topo II activity is not required for structural maintenance of mitotic chromosomes. Several treatments were performed before (a, c, and e) or after (b, d, and f) chromosome assembly. (a, c, and e); sperm chromatin was incubated with mitotic extracts containing control rabbit IgG (10 μ g/ml; a), anti-topo II IgG (10 μ g/ml; c), and VM-26 (10 μ M; e). After a 2-h incubation, chromosomes were fixed and stained with Hoechst 33258. We obtained similar results when the extracts were pretreated 30 min before the addition of sperm chromatin. (b, d, and f); mitotic chromosomes were assembled by incubating sperm chromatin with an untreated mitotic extract for 2 h. The reaction mixture was divided into three aliquots, and control rabbit IgG (10 μ g/ml; b), anti-topo II IgG (10 μ g/ml; d), and VM-26 (50 μ M; f) were added. After another 1-h incubation, chromosomes were fixed and stained as above. Bar, 10 μ m.

and Laemmli, 1987; Adachi et al., 1991). If topo II activity is continuously required to organize the chromatin loops, the loss of topo II activity may induce global effects on the morphology of mitotic chromosomes. To test this possibility, we added affinity-purified anti-topo II IgG into reaction mixtures. A concentration of affinity-purified anti-topo II IgG (10 μ g/ml) efficiently blocked chromosome assembly and condensation when added before assembly (Fig. 6 c). However, once condensation was completed, the same concentration of the antibody did not induce any detectable changes on the morphology of condensed chromosomes (Fig. 6 d). To rule out a possibility that the antibody may not be accessible to all topo II molecules associated with chromosomes, a topo II inhibitor was also tested. The addition of a topo II inhibitor VM-26 has similar effects to those observed by the addition of anti-topo II IgG: as low as 10 μ M of VM-26 is sufficient to block local condensation that follows chromatin swelling (Fig. 6 e), whereas even high concentration of the same drug (50–100 μ M) induced no remarkable changes on the structure of precondensed chromosomes (Fig. 6 f).

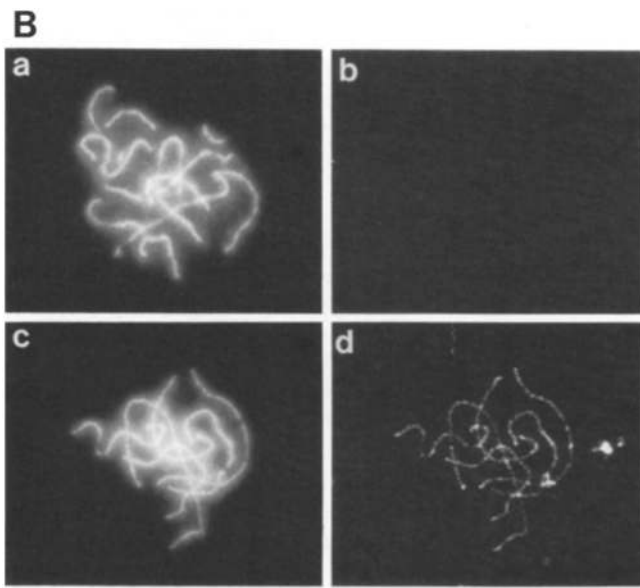
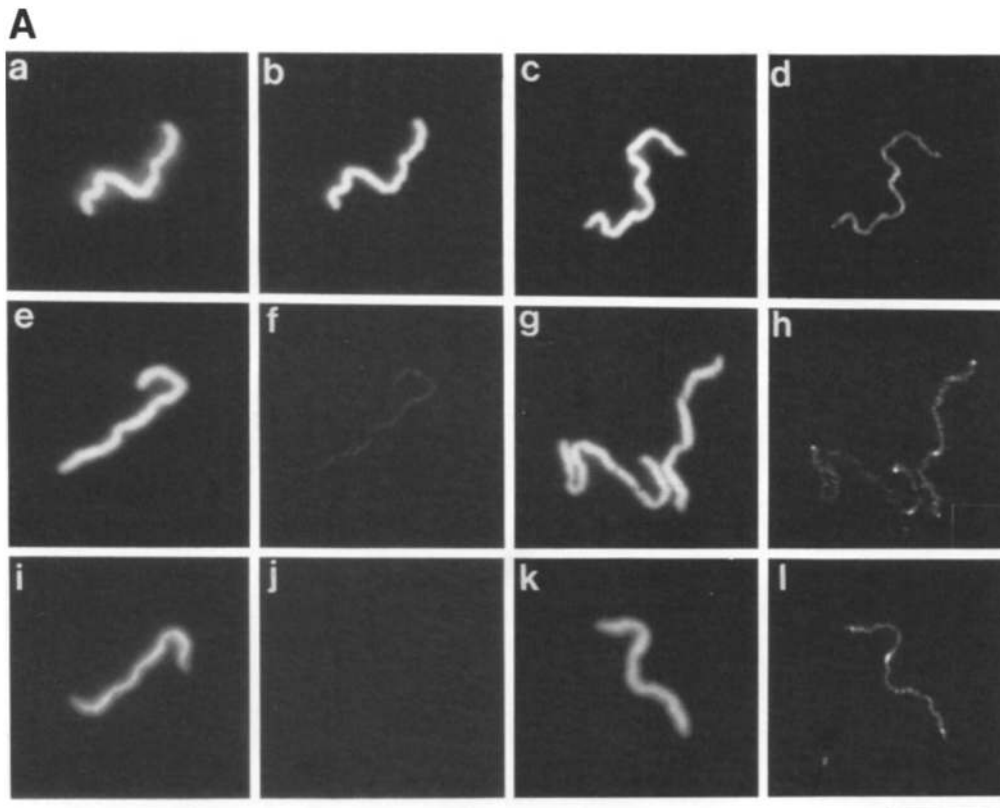


Figure 7. Topo II is dissociated from mitotic chromosomes under mild conditions. (A) Salt extraction. Mitotic chromosomes were assembled from sperm chromatin *in vitro*, and then mixed with an equal vol of EB containing increasing concentrations of NaCl. The extra concentrations of NaCl were 0 mM (a–d), 50 mM (e–h) and 100 mM (i–l). After incubation at 23°C for 10 min, chromosomes were fixed and stained with Hoechst 33258 (a, c, e, g, i, and k), affinity-purified anti-topo II (b, f, and j) and MPM-2 (d, h, and l). Bar, 10 μm. (B) Dextran sulfate extraction. Mitotic chromosomes were assembled from sperm chromatin *in vitro*, and then dextran sulfate was added to a final concentration of 100 μg/ml. Chromosomes were fixed and stained with Hoechst 33258 (a and c), affinity-purified anti-topo II (b), and MPM-2 (d). Bar, 10 μm.

These results suggest that continuous catalytic activity of topo II may not be required for the structural maintenance of mitotic chromosomes once their condensation is completed.

Topo II Is Dissociated from Mitotic Chromosomes under Mild Conditions

To test further a possible scaffolding role of topo II, we next attempted to extract mitotic chromosomes assembled in our system. Conventional procedures for scaffold preparation involve a number of experimental steps including chromosome

isolation from drug-arrested mitotic cells, scaffold stabilization, and histone extraction. Any of these steps could potentially induce nonphysiological effects on the chromosome morphology. In contrast, in our *in vitro* assembly system, it is possible to manipulate chromosomes directly in the assembly mixtures without any isolation or stabilization steps.

Surprisingly, we found that topo II can be extracted from mitotic chromosomes under mild conditions where the morphology of the chromosomes is well preserved. Mitotic chromosomes were assembled in EB (80 mM β-glycerophosphate, pH 7.3, 15 mM MgCl₂, 20 mM EGTA and 1 mM

DTT with protease inhibitors) under the standard assembly condition and then mixed with the same buffer containing increasing concentrations of NaCl. When extra concentration of NaCl in an assembly mixture was raised to 50 mM, the rod-shaped chromosomes expanded slightly as judged by Hoechst staining (Fig. 7 A, e and g). At 100 mM, the chromatin expanded further to form a small halo around a central axis of each chromosome (Fig. 7 A, i and k). These structural changes were highly uniform and reproducible. Under these relatively mild conditions, topo II molecules were dissociated from the mitotic chromosomes. By immunofluorescence, topo II staining was greatly reduced at 50 mM NaCl and residual, faint signal was observed restricted on a central axis of the expanded chromosomes (Fig. 7 A, f). No detectable signal was observed at 100 mM NaCl, indicating that topo II was completely extracted at this salt concentration (Fig. 7 A, j). In contrast to topo II, MPM-2 antigens were relatively resistant to the same treatments. Fluorescent intensity on the axis was slightly reduced but the discrete staining pattern did not change (Fig. 7 A, h and l). Interestingly, we noted that MPM-2 also stained tips at both ends of chromosomes and a patch located on the central axis in the expanded chromosomes. Presumably these correspond to telomeres and the kinetochores, respectively. While these structures are present in unextracted chromosomes, they were clearly visualized on chromosomes extracted at extra 50 mM NaCl due to a slight reduction of total staining intensity on the central axis (Fig. 7 A, h).

A different extraction procedure also efficiently extracted topo II molecules from mitotic chromosomes assembled *in vitro*. Dextran sulfate, a negatively charged polymer, has been used to extract chromosomal proteins by competition with DNA in a low ionic strength condition (Lewis and Laemmli, 1982). When dextran sulfate was added at a final concentration of 100 $\mu\text{g/ml}$ into an assembly mixture after condensation was completed, the length of the chromosomes increased substantially (at least twofold) and the chromatin swelled to form a large halo around a central axis. Under these conditions the chromosomes tended to aggregate, but individual chromosomes were clearly visualized (Fig. 7 B, a and c). When these structures were stained with anti-topo II, no immunofluorescent signal was detected (Fig. 7 B, b). In contrast, MPM-2 strongly stained the axis but not the halo (Fig. 7 B, d).

Immunoblot analysis confirmed that topo II polypeptides were dissociated from mitotic chromosomes under these extraction conditions. Control and extracted chromosomes were partially purified by centrifugation, and the amounts of topo II coprecipitated with chromosomes were analyzed by immunoblotting (Fig. 8). Consistent with immunofluorescent data, no detectable level of topo II (<2% of the unextracted control) was found in the pellet when treated with extra 0.1 M NaCl or 100 $\mu\text{g/ml}$ dextran sulfate, although chromosomal DNA was quantitatively recovered under these conditions (data not shown).

Since chromosome organization and axial structures are maintained after topo II extraction, we conclude that topo II does not play a scaffolding role in the structural maintenance of mitotic chromosomes assembled in this system.

Changes of Topo II Distribution under Artificial Conditions

Although topo II molecules were completely extracted from

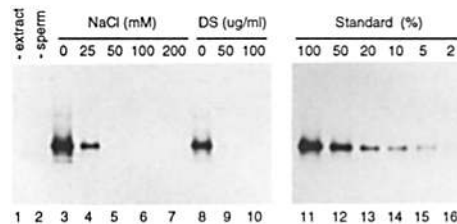


Figure 8. Immunoblot analysis of topo II extraction. Sperm chromatin was incubated with mitotic extracts in the standard condition (lanes 3–10) for 2 h. As negative controls, no mitotic extract (lane 1) or no sperm chromatin (lane 2) was added. Assembled chromosomes were treated with NaCl (lanes 3–7) or dextran sulfate (lanes 8–10) at final concentrations as indicated and then partially purified by centrifugation. Topo II molecules coprecipitated with the chromosomes were analyzed by immunoblotting. A dilution series of the untreated chromosome sample (equivalent to lane 3) was loaded in the same gel as standards (*standard*; lanes 11–16).

mitotic chromosomes with extra 0.1 M NaCl (Fig. 7 A, j), there was a tendency that, in a milder condition, residual topo II was found on the chromosomal axis (Fig. 7 A, f). This observation suggested that topo II may have higher affinity for the axial region of chromatin compared with peripheral region. To test this possibility, we screened several conditions that might affect the distribution of topo II in mitotic chromosomes (Table I).

When EtBr (ethidium bromide) was added at a final concentration of 50 $\mu\text{g/ml}$, the rod-shaped chromosomes expanded slightly (Fig. 9 c) and topo II was found concentrated on an axial region (Fig. 9 d). At higher concentration of EtBr (100 $\mu\text{g/ml}$), the central axis was surrounded by a large halo of chromatin (Fig. 9 e). In these chromosomes, total fluorescent signal with anti-topo II was greatly reduced and the residual topo II was restricted to the axis (Fig. 9 f). Since it is known that EtBr intercalates into DNA and unwinds double-stranded DNA, this result suggests that changes of DNA topology may induce a gross rearrangement of topo II distribution in mitotic chromosomes.

Copper ion is known to specifically stabilize the scaffold structure of isolated chromosomes (Lewis and Laemmli, 1982). We found that addition of CuSO_4 at a final concentration of 0.5 mM induced a slight expansion of rod-shaped chromosomes (Fig. 9 g). In these expanded chromosomes, topo II was not uniformly distributed but was found concentrated on an axial region (Fig. 9 h). The effect was Cu^{2+} specific: any divalent cation other than Cu^{2+} we have tested did not induce either the expansion of chromosomes or the change of topo II distribution (Table I). We attempted chromosome extraction with extra 0.1 M NaCl after these pretreatments, but failed to find a condition that causes tight association of topo II with the chromosomes (Table I).

Discussion

Mitotic Chromosome Assembly from Sperm Chromatin *In Vitro*

Upon fertilization, *in vivo*, sperm chromatin is induced to form the male pronucleus, and then is converted into mitotic chromosomes after the fusion with the female nucleus. Therefore, the “mitotic” pathway in which sperm chromatin is directly induced to produce condensed chromosomes is less physiological than the “interphase-to-mitotic” pathway in

Table I. Effects of Various Treatments on Chromosome Morphology and Topo II Localization

Pretreatments	No extraction		0.1 M NaCl extraction	
	Chromosome morphology	Topo II staining	Chromosome morphology	Topo II staining
No treatment	Rod	++	Halo	-
50 μ g/ml EtBr	Expanded	++ (axis)	Large halo	-
100 μ g/ml EtBr	Halo	+ (axis)	Large halo	\pm (axis)
0.5 mM CuSO ₄	Expanded	++ (axis)	Large halo	-
0.5 mM MgSO ₄	Rod	++	Halo	-
0.5 mM ZnSO ₄	Rod	++	Halo	-
0.5 mM MnSO ₄	Rod	++	Halo	-
0.5 mM CoCl ₂	Rod	++	Halo	-
0.5 mM CaCl ₂	Rod	++	Halo	-
0.5 mM CuSO ₄ (37°C)	Expanded	++ (axis)	Large halo	\pm (axis)
0.5 mM CaCl ₂ (37°C)	Rod	++	Halo	-
37°C	Rod	++	Halo	-
20 mM EDTA	Rod	+	Halo	\pm (axis)

Mitotic chromosomes were assembled from sperm chromatin in the standard condition at 23°C for 2 h. Pretreatments were performed at 23 or 37°C for 10 min. The reaction mixtures were divided into two aliquots and then mixed with an equal vol of EB (no extraction) or EB containing 0.2 M NaCl (0.1 M NaCl extraction). After incubation at 23°C for 10 min, chromosome morphology and topo II localization was examined by immunofluorescence. The intensity of topo II staining was based on the examination by eye and is not quantitative. Chromosomes were uniformly stained with anti-topo II unless otherwise indicated.

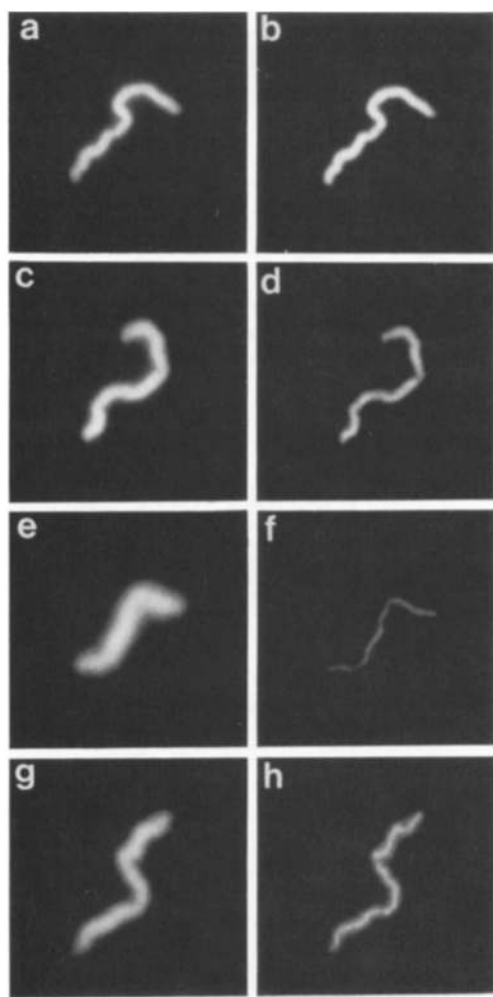


Figure 9. Artfactual changes of topo II distribution in mitotic chromosomes. Mitotic chromosomes were assembled from sperm chromatin in vitro in the standard condition and then EtBr (a and b, 0 μ g/ml; c and d, 50 μ g/ml; e and f, 100 μ g/ml) or CuSO₄ (g and h, 0.5 mM) was added. After 10 min incubation, chromosomes were fixed and stained with Hoechst 33258 (a, c, e, and g) and affinity-purified anti-topo II IgG (b, d, f, and h). Bar, 10 μ m.

which mitotic chromosomes are produced from interphase nuclei containing replicated DNA. However, even the former pathway examined in this study produces mitotic chromosomes with a good morphology and a final condensation state similar to that observed in vivo. Thus results obtained with this assay for studying the mechanism of chromosome condensation are relevant to the physiological mechanism. We have investigated in detail the structural rearrangements of sperm chromatin observed in mitotic extracts and characterized the four intermediate stages as follows: (a) swelling of highly compact sperm chromatin; (b) local condensation; (c) formation of entangled thin fibers; and (d) resolution of highly condensed chromosomes. The thin chromatin fibers observed in stage c have a diameter of \sim 0.2–0.4 μ m, presumably corresponding to prophase chromosomes. The final condensed products observed in stage d with a diameter of \sim 0.8 μ m may represent fully condensed metaphase chromatids. Our impression from fluorescence morphology is that the last step of condensation appears to involve coiling of the thin fibers (Fig. 1, d and e). However, more extensive morphological studies will be needed to clarify the structural details of the condensation processes in this system.

Role of Topo II in Mitotic Chromosome Assembly and Organization In Vitro

It is well known that sperm chromatin mostly consists of sperm-specific chromatin proteins that are replaced by embryonic ones upon fertilization (reviewed by Poccia, 1989). It is therefore possible to investigate specific function of non-sperm chromatin components recruited from extracts under a physiological condition in our system. Since we have found that *Xenopus* sperm chromatin lacks detectable amounts of both isotypes of endogenous topo II (α and β), the α type present in the extracts is the only source of topo II molecules to be considered for mitotic chromosome assembly and condensation in vitro. Our depletion and complementation experiments have clearly demonstrated that topo II (α) is required for chromosome assembly and condensation that follows swelling of sperm chromatin. This is in good agreement with Adachi et al. (1991), who used chicken erythrocyte nuclei as a substrate with a low level of endogenous topo

II in a similar condensation assay. There is, however, one significant difference. Adachi et al. (1991) observed that in topo II-depleted extracts erythrocyte nuclei are converted into partially resolved, uncondensed chromatids called "precondensation chromosomes" (pcXs), whereas we observed no indication of resolution in the swollen sperm chromatin in the absence of topo II. At the moment we do not know if the discrepancy is due to either the different condensation mechanisms or the different protein composition in the nuclei used for these two studies.

It was not clear if topo II has a structural role in the maintenance of mitotic chromosomes from previous studies in vitro (Newport and Spann, 1987; Wood and Earnshaw, 1990; Adachi et al., 1991). Adachi et al. (1991) argued in favor of structural involvement of topo II based on their dosage experiment: a certain dose of topo II in an extract induced partial but not complete condensation even after longer incubation, leading to the suggestion that the reaction is not exclusively catalytic. Since we have found that topo II is indeed associated with mitotic chromosomes assembled in vitro, one would expect our condensation system to reflect a structural requirement for topo II function. In principle, two types of structural requirement could be considered. Topo II may be required as part of an axial, scaffold structure, or alternatively it could play a more diffuse structural role in chromosome condensation. We presented two lines of evidence to suggest that topo II does not play a scaffolding role in mitotic chromosome organization in our system. First, once condensation is completed, inhibition of topo II activity has little effect on the morphology of condensed chromosomes. Second and more convincing evidence is that topo II molecules can be dissociated from mitotic chromosomes under mild conditions where the shape of chromosomes is well preserved and the condensation state is little altered, particularly along the length of the chromosomes. We obtained identical results using two different extraction procedures, either by increasing ionic strength or by adding a polyanion to assembly mixtures. All detectable topo II molecules were extracted in both conditions, whereas other chromosomal antigens recognized with a mAb MPM-2 remained associated with a central axis of chromosomes. Although we cannot rule out the possibility that topo II also has a low level of MPM-2 epitope, the differential extraction pattern suggests that the predominant chromosomal MPM-2 antigens are distinct from topo II.

Since one could argue that our analyses may not be sensitive enough to detect small, but significant amounts of topo II remaining in the extracted chromosomes, it is important to estimate the stoichiometry of topo II in mitotic chromosomes. Based on the DNA concentration of sperm chromatin added into extracts and the amount of topo II coprecipitated with condensed chromosomes (as judged by quantitative immunoblotting), we estimate that topo II/DNA ratio is 5–10 kb per topo II dimer in the unextracted chromosomes. When extracted with extra 0.1 M NaCl, the amount of residual topo II was <2% of the unextracted control, thus giving a ratio of not less than 250–500 kb per topo II dimer. This value is far beyond the topo II level in isolated chromosomes (~50 kb per topo II dimer; Gasser et al., 1986).

Thus our data argue that while a chromosome scaffold may exist, topo II is not required for its structural maintenance in this system at least once condensation is completed. How-

ever, we cannot rule out the possibility that topo II acts as a "loop fastener" in early stages of chromosome assembly. Our data do not address whether topo II plays a more diffuse structural role in maintaining condensation. When topo II was extracted, slight swelling always occurred, but we cannot tell whether this was due to extraction of topo II or some other chromosomal proteins.

Factors Affecting Topo II Localization in Mitotic Chromosomes Assembled In Vitro

Our immunofluorescence data showed uniform distribution of topo II on condensed chromosomes, but in particular extraction conditions residual topo II was found concentrated on a central axis (e.g., Figs. 7 A, f and 9 f). Topo II may in fact have high affinity for the axial region of chromatin compared with peripheral regions. EtBr treatments of condensed chromosomes suggested that changes of DNA topology potentially induce partial release or redistribution of topo II. A recent finding that topo II has high affinity for supercoiled DNA by preferentially interacting with DNA cross-overs (Zechiedrich and Osheroff, 1990) may account for our observation. Since torsional stress of DNA introduced by intercalation of EtBr is removed by topo I present in the assembly mixtures, topologically relaxed chromatin loops become expanded and thus lose their affinity for topo II. In contrast, the axial chromatin may be relatively resistant to the treatment and maintains a high density of DNA cross-overs that provide high affinity binding sites for topo II. Another factor that potentially affects topo II localization is copper ion, which has been used for the stabilization of the chromosome scaffold structure (Lewis and Laemmli, 1982). When mitotic chromosomes are incubated with 0.5 mM CuSO₄, topo II was found concentrated on an axial region although the mechanism of this effect is not clear. We also found that several pretreatments of chromosomes affect the efficiency of topo II extraction with extra 0.1 M NaCl (Table I), but even in these conditions the level of residual topo II was <5% of an unextracted control as judged by quantitative immunoblotting. It should be emphasized that all these treatments affecting the behavior of topo II, when applied before assembly, prevent chromosome condensation in our system (data not shown), and thus seem to be nonphysiological.

In Vivo Chromosomes vs In Vitro Chromosomes

Previous morphological and biochemical analyses of mitotic chromosomes isolated from somatic tissue culture cells suggested that topo II is an integral scaffold component required for the maintenance of mitotic chromosome structure (Earnshaw et al., 1985; Gasser et al., 1986). Inconsistent with this notion, our results obtained from an in vitro assembly system have shown that topo II does not play a scaffolding role at least once condensation is completed. While it is not easy to reconcile the discrepancy of these studies at present, it will be important to clarify the potential differences between the two systems and to evaluate their characteristics carefully. One important question is the extent to which chromosome assembly achieved in *Xenopus* egg extracts strictly mimics that observed in somatic cells. It has been suggested that some chromosomal events in the early embryonic cell cycle may be significantly different from those in the somatic one. These include mechanisms of nucleosome assembly

and sequence requirements for initiation of DNA replication (reviewed by Laskey and Leno, 1990). Extensive morphological and biochemical analyses will be required to answer this question and our results will be most relevant to embryonic chromosome structures. Despite this potential problem, dissection of mitotic chromosomes assembled *in vitro* has a major advantage over the previous studies using chromosomes isolated from somatic cells. As demonstrated in this paper, we can manipulate "native" chromosomes in their assembly condition without any isolation and stabilization steps. In principle, any procedures for chromosome isolation may result in structural changes. Even colcemid treatment of mitotic cells can cause morphological changes of chromosomes (reviewed by Rieder and Palazzo, 1992) and a relocalization of some chromosomal antigens (Earnshaw and Cooke, 1991; Compton et al., 1991; 1992). It should be noted that in previous studies topo II was localized on a central axis only when mitotic chromosomes were experimentally swollen or expanded (Earnshaw and Heck, 1985; Gasser et al., 1986). These studies, although they paid careful attention to maintaining a native state of chromosomes, should be re-evaluated because our data implies that even mild treatments potentially induce partial release or relocalization of topo II molecules in mitotic chromosomes.

In conclusion, our *in vitro* system provides a complementary approach to the conventional studies dissecting mitotic chromosomes isolated from somatic cells. Comparative evaluation of the results obtained from the two systems will contribute to our comprehensive understanding of mitotic chromosome structure and organization.

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