

A role of topoisomerase II in linking DNA replication to chromosome condensation

Olivier Cuvier and Tatsuya Hirano

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724

The condensin complex and topoisomerase II (topo II) have different biochemical activities *in vitro*, and both are required for mitotic chromosome condensation. We have used *Xenopus* egg extracts to investigate the functional interplay between condensin and topo II in chromosome condensation. When unreplicated chromatin is directly converted into chromosomes with single chromatids, the two proteins must function together, although they are independently targeted to chromosomes. In contrast, the requirement for topo II is temporarily separable from that of condensin when chromosome assembly is induced after DNA replication. This experimental setting allows us to find that, in

the absence of condensin, topo II becomes enriched in an axial structure within uncondensed chromatin. Subsequent addition of condensin converts this structure into mitotic chromosomes in an ATP hydrolysis-dependent manner. Strikingly, preventing DNA replication by the addition of geminin or aphidicolin disturbs the formation of topo II-containing axes and alters the binding property of topo II with chromatin. Our results suggest that topo II plays an important role in an early stage of chromosome condensation, and that this function of topo II is tightly coupled with prior DNA replication.

Introduction

Chromosome condensation ensures the faithful segregation of the genetic information in mitosis. In eukaryotes, this fundamental cellular process involves a highly coordinated folding of the chromatin fiber into mitotic chromosomes, a process that remains poorly understood at the molecular level. Accumulating lines of genetic and biochemical evidence suggest that a large protein complex, called condensin, plays a crucial role in mitotic chromosome assembly and organization (for review see Cobbe and Heck, 2000; Hirano, 2002). Condensin is highly conserved from yeast to humans, and is composed of two structural maintenance of chromosomes (SMC)* ATPase subunits and three non-SMC subunits. Purified condensin has the ability to introduce superhelical tension into DNA in an ATP-dependent manner *in vitro* (Kimura and Hirano, 1997; Kimura et al., 1999, 2001; Hagstrom et al., 2002). This activity involves the formation of two oriented gyres of DNA around a single condensin complex, and most likely relies on the unique two-armed structure of the SMC core subunits (Bazzett-Jones et al.,

2002). The non-SMC subunits bind to the ATPase domains of the SMC heterodimer, and regulate its ATPase activity and mode of interaction with DNA (Kimura and Hirano, 2000; Anderson et al., 2002; Yoshimura et al., 2002). It is unknown how the *in vitro* activities of condensin might contribute to chromosome condensation in the cell, or whether condensin might have an additional architectural role in organizing higher order chromosome structure.

Topoisomerase II (topo II), which catalyzes a transient breakage and reunion of double-stranded DNA, was the first protein shown to be essential for mitotic chromosome condensation (Uemura et al., 1987). A role for topo II in the structural maintenance of mitotic chromosomes has been suggested on the basis of the finding that topo II is a major constituent of the chromosome scaffold (Earnshaw et al., 1985; Gasser et al., 1986). A study using a *Xenopus* egg cell-free extract demonstrated a stoichiometric contribution of topo II to chromosome assembly (Adachi et al., 1991). However, the exact role of topo II in chromosome organization remains controversial because different approaches failed to detect a stable association of topo II with mitotic chromosomes (Hirano and Mitchison, 1993; Swedlow et al., 1993). Moreover, recent studies have shown that the association of topo II with chromosomes in living cells appears to be more dynamic than predicted before (Christensen et al., 2002; Tavormina et al., 2002).

One prominent phenotype of condensin mutants is a defect in chromosome segregation in anaphase (Saka et al., 1994;

Address correspondence to Tatsuya Hirano, Cold Spring Harbor Laboratory, 1 Bungtown Rd., Cold Spring Harbor, NY 11724. Tel.: (516) 367-8370. Fax: (516) 367-8815. E-mail: hirano@cshl.org

*Abbreviations used in this paper: HSS, high speed supernatant; LSS, low speed supernatant; SMC, structural maintenance of chromosomes; topo II, topoisomerase II.

Key words: *Xenopus* egg extracts; condensin; decatenation; compaction; SMC

Strunnikov et al., 1995; Sutani et al., 1999; Steffensen et al., 2001; Bhalla et al., 2002; Hagstrom et al., 2002). This is reminiscent of (if not identical to) the phenotype observed in topo II mutants. On the basis of these observations and other mechanistic considerations, it has been proposed that one of the important roles of chromosome condensation is to assist topo II-mediated decatenation of sister chromatids (Koshland and Strunnikov, 1996; Hirano, 2000; Holmes and Cozzarelli, 2000). Consistent with this notion, it was reported that a regulatory subunit of condensin (Barren) interacts directly with topo II in *Drosophila* (Bhat et al., 1996), and that a different subunit (YCS4p) is required for the binding of topo II to chromatin in *Saccharomyces cerevisiae* (Bhalla et al., 2002). However, other studies did not detect a direct interaction between the two proteins or their interdependent loading onto chromosomes (Hirano et al., 1997; Lavoie et al., 2000). Thus, it remains to be established exactly how condensin and topo II might cooperate to support chromosome condensation and segregation.

In this work, we have used *Xenopus* egg extracts to study the functional interactions between condensin and topo II in mitotic chromosome condensation. We find that the presence or absence of DNA replication affects the modes of action of topo II in chromosome condensation during subsequent mitosis. Only when mitosis is induced after DNA replication, topo II becomes concentrated on an axial structure that can be visualized in the absence of condensin. This subchromosomal structure can then be converted into mitotic chromosomes by subsequent action of condensin. We propose that topo II has a previously uncharacterized role in linking DNA replication to mitotic chromosome condensation.

Results

Topo II and condensin play distinct roles in mitotic chromosome assembly

To address the precise roles and potential collaboration of condensin and topo II in mitotic chromosome assembly, each of the two proteins was immunodepleted from a mitotic high speed supernatant (HSS) of the *Xenopus* egg extract. As judged by quantitative immunoblotting, more than 98% of condensin or topo II was depleted under these conditions (Fig. 1 A, lanes 2 and 8). Consistent with the previous study (Hirano et al., 1997), depletion of either condensin or topo II did not affect the level of the other one in the extract (Fig. 1 A, lanes 13–15), suggesting that these proteins do not associate with each other before being targeted to chromatin.

When sperm chromatin was incubated with the mock-depleted extract, it was efficiently converted into a cluster of rod-shaped chromosomes (Fig. 1 B, a). In the condensin-depleted extract, chromatin with a round-shaped appearance was observed, and there was no sign of the formation of mitotic chromosomes (Fig. 1 B, b; Hirano et al., 1997). Immunodepletion of topo II also caused a defect in chromosome assembly, but its defective phenotype was distinct from that observed in the absence of condensin (Fig. 1 B, c; Hirano and Mitchison, 1993). To dissect these structures in more detail, the assembled chromatin was treated with buffers

containing increasing concentrations of NaCl (Fig. 1 B, d–f and g–i). Salt treatment of the chromosomes assembled in the mock-depleted extract loosened their structure, producing a more extended appearance surrounded by a halo of DNA (Fig. 1 B, d and g). The chromatin formed in the condensin-depleted extract was very fragile and was easily broken apart on salt treatment (Fig. 1 B, e and h), whereas the elongated chromatin mass assembled in the absence of topo II was relatively resistant to salt (Fig. 1 B, f and i). Immunofluorescent staining showed that condensin was loaded onto chromatin in the absence of topo II (Fig. 1 C, d), and conversely, topo II could bind to chromatin in the condensin-depleted extract (Fig. 1 C, h).

To test whether condensin might affect topo II activities in the extracts, we used two different assays. In the first one, the topo II-specific inhibitor VM-26 was added to measure topo II-mediated cleavage of plasmid DNA (Fig. 1 D, left), and in the second, kinetoplast DNA was used to measure the decatenation activity of topo II (Fig. 1 D, middle). We found that neither of the two activities was affected in the absence of condensin, although they were barely detectable when topo II was depleted from the extracts. Topo II-mediated DNA cleavage was also tested in the context of sperm chromatin (Fig. 1 D, right). Again, it was not affected by depletion of condensin. These results show that condensin and topo II have distinct roles in chromosome assembly in the cell-free extracts.

Topo II and condensin must function simultaneously when chromosome assembly is induced from unreplicated substrates

Next, we tested whether condensin and topo II must function simultaneously in mitotic chromosome assembly or whether their actions are temporarily separable. To this end, we set up a stepwise protocol for chromosome assembly. Sperm chromatin was first incubated with condensin-depleted interphase HSS, and then with mitotic HSS that had also been depleted of condensin. Finally, condensin was supplemented by adding topo II-depleted mitotic HSS into the assembly mixture (Fig. 2 A, protocol 1). Rod-shaped chromosomes were successfully assembled under this condition (Fig. 2 B, a–c). Because HSS supports no DNA replication (Fig. 2 B, d), the assembled chromosomes were composed of single chromatids. In contrast, when the topo II inhibitor VM-26 was added before allowing condensin to function (Fig. 2 A, protocol 2), chromosome assembly was severely impaired and no individual chromosome was observed (Fig. 2 B, e–g). These results suggest that topo II and condensin must work simultaneously to assemble mitotic chromosomes under this condition, although the two proteins play distinct roles in this process.

Temporal requirements for topo II and condensin are separable when chromosome assembly is induced after DNA replication

The experiments described above were performed using HSS in which chromosomes are assembled without preceding DNA replication (Hirano and Mitchison, 1993). To further investigate the roles of topo II and condensin in

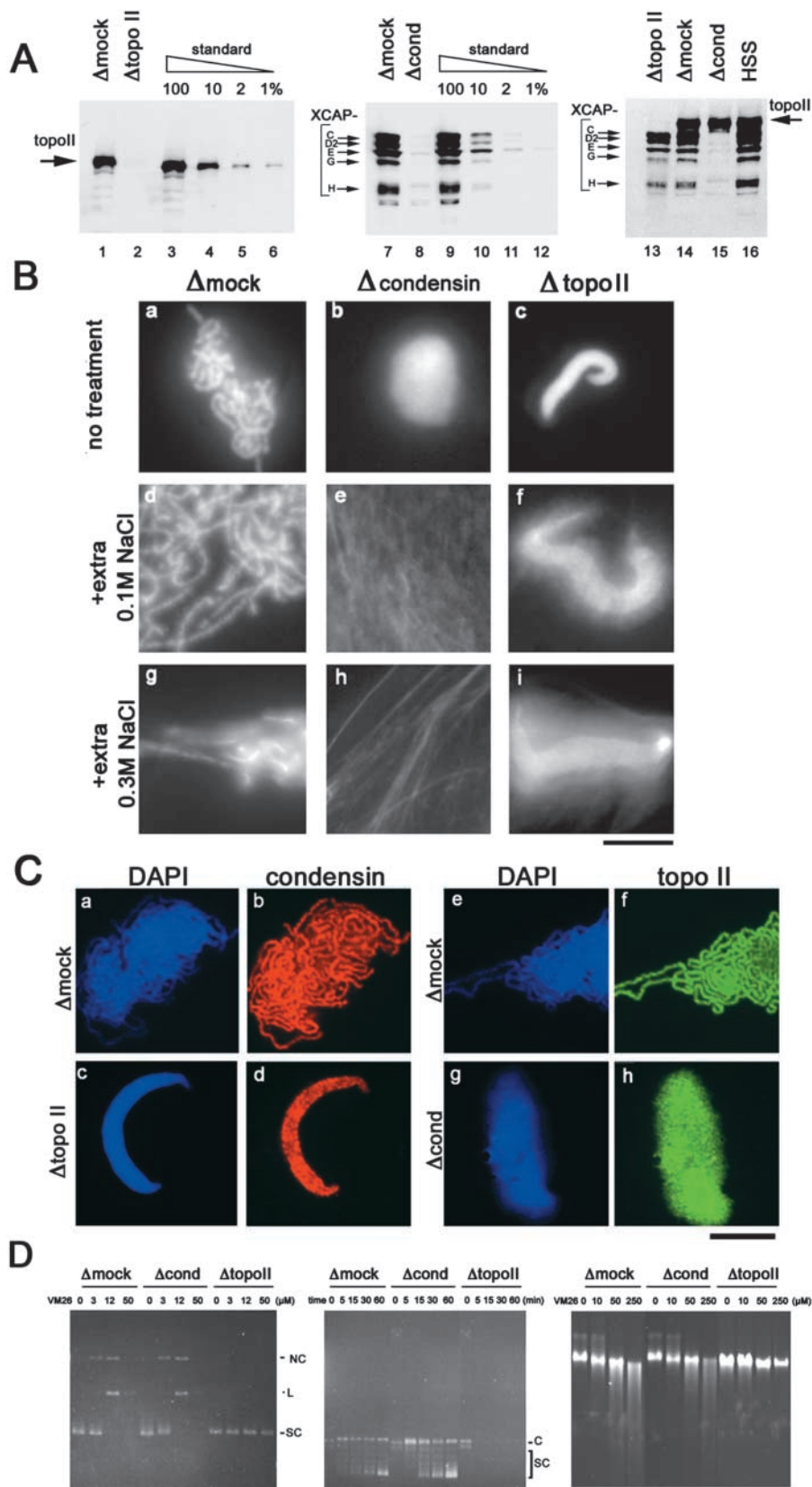


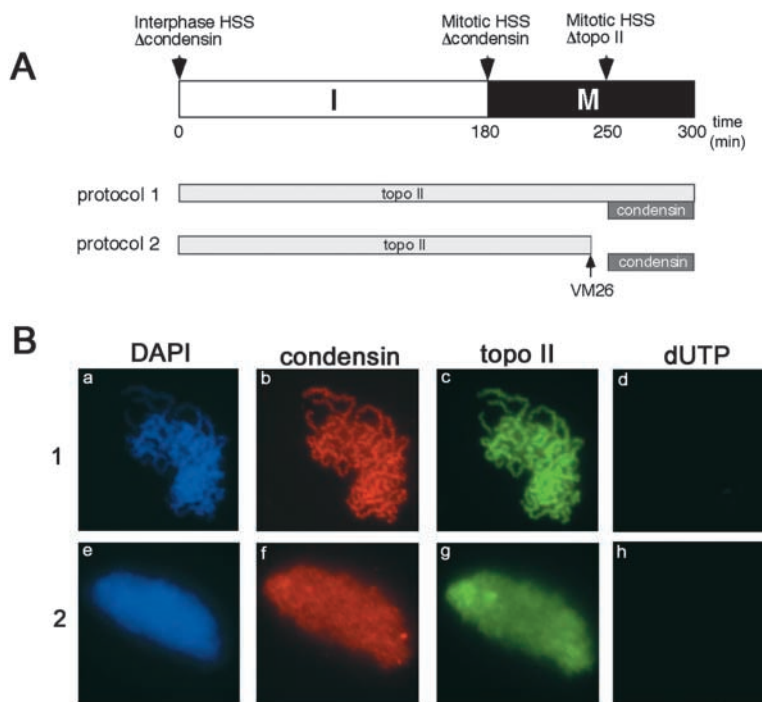
Figure 1. Condensin and topo II play distinct roles in chromosome assembly.

(A) A mitotic HSS was immunodepleted with an anti-topo II (lanes 2 and 13) antiserum or a mixture of affinity-purified condensin antibodies (lanes 8 and 15). Mock-depleted extracts were prepared using nonimmune antiserum (lane 1) or IgG (lanes 7 and 14). Equal volumes of these extracts were subjected to 7.5% SDS-PAGE, blotted onto a nitrocellulose paper, and probed with the anti-topo II (lanes 1–6), a mixture of anti-condensin antibodies (lanes 7–12), or both (lanes 13–16). A dilution series of untreated extract (lanes 3–6 and 9–12) was analyzed in parallel to estimate the efficiency of immunodepletion. (B) Chromosomes were assembled from sperm chromatin in mock-depleted (a, d, and g), condensin-depleted (b, e, and h), or topo II-depleted (c, f, and i) HSS. After assembly, the mixture was treated with the indicated extra concentrations of NaCl for 20 min at 22°C, fixed, and stained with DAPI. Bar, 10 μ m. (C) Chromosomes were assembled in mock-depleted (a, b, e, and f) topo II-depleted (c and d) or condensin-depleted (g and h) HSS, and immunostained with anti-condensin (XCAP-G; b and d) or anti-topo II antibody (f and h). DNA was counterstained with DAPI (a, c, e, and g). Bar, 10 μ m. (D) Topo II assays. (Left) Supercoiled DNA was incubated in the control or depleted extracts in the presence of an increasing concentration of VM-26. The DNA was purified, fractionated on a 0.7% agarose gel, and stained with EtBr. The positions of circular (C), nicked-circular (NC), linear (L), and supercoiled (SC) DNA are indicated. (Middle) Kinetoplast DNA was incubated with the extracts and the reaction was terminated at the indicated time points. The DNA was deproteinized and analyzed on a 0.8% agarose gel. In the mock- and condensin-depleted extracts, kinetoplast DNA was rapidly decatenated to produce relaxed minicircles that became supercoiled as a result of nucleosome assembly. In the topo II-depleted extract, the catenated DNA remained in the well and was not resolved in the gel. (Right) Sperm chromatin was incubated with the depleted extracts to allow chromosome assembly. An increasing concentration of VM-26 (as indicated) was then added to induce topo II-mediated DNA cleavage.

this process, we used low speed supernatants (LSS) that allow one complete round of DNA replication before entry into mitosis (Blow and Laskey, 1986; Murray, 1991). The experimental setting depicted in Fig. 3 A was essentially the

same as that used in Fig. 2. Depletion of condensin had little effect on the kinetics of nuclear envelope assembly in interphase LSS as judged by phase contrast microscopy (unpublished data). Mitosis was induced in the absence of

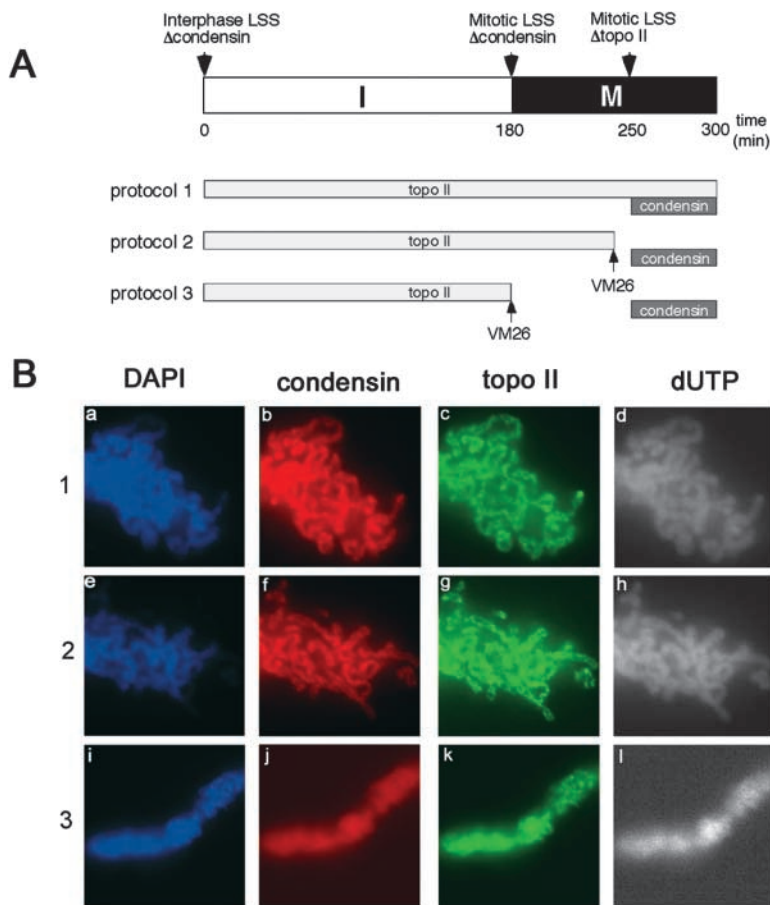
Figure 2. Topo II and condensin must function simultaneously to assemble chromosomes in the absence of preceding DNA replication. (A) Experimental scheme. Sperm chromatin was incubated with interphase HSS that had been immunodepleted of condensin. After incubation at 22°C for 180 min, the assembly mixture was converted into mitosis by adding condensin-depleted mitotic HSS. After another 60 min (at 240 min), the mixture was split into two aliquots; one was treated with no drug (protocol 1), and the other was treated with VM-26 at a final concentration of 10 μ M (protocol 2). At 250 min, topo II-depleted mitotic HSS was added to allow condensin to function. (B) Chromosomes were assembled according to protocol 1 (a–d) or protocol 2 (e–h), fixed at 300 min, and stained with anti-condensin (XCAP-G; b and f) and anti-topo II antibodies (c and g). DNA was counterstained with DAPI (a and e), and DNA replication was assayed with incorporation of biotinylated dUTP (d and h). Bar, 10 μ m.



condensin, and then condensin was supplemented by adding topo II–depleted mitotic LSS (Fig. 3 A, protocol 1). This protocol produced mitotic chromosomes with duplicated sister chromatids (Fig. 3 B, a–c). Complete duplica-

tion of chromosomal DNA was confirmed by a uniform incorporation of biotinylated dUTP (Fig. 3 B, d). When topo II activity was blocked with VM-26 before adding condensin (Fig. 3 A, protocol 2), we found that similar, if

Figure 3. Temporal requirements for topo II and condensin are separable when chromosome assembly is induced after DNA replication. (A) Essentially the same procedure was used as in Fig. 2 A, except that LSS were used instead of HSS. No VM-26 was added in protocol 1. VM-26 was added at 240 min (60 min after mitotic entry) in protocol 2, or at 180 min (at the onset of mitosis) in protocol 3. (B) Chromosomes were assembled according to protocol 1 (a–d), protocol 2 (e–h), or protocol 3 (i–l), fixed at 300 min, and stained with anti-condensin (XCAP-G; b, f, and j) and anti-topo II antibodies (c, g, and k). DNA was counterstained with DAPI (a, e, and i), and DNA replication was assayed with incorporation of biotinylated dUTP (d, h, and l). Bar, 10 μ m.



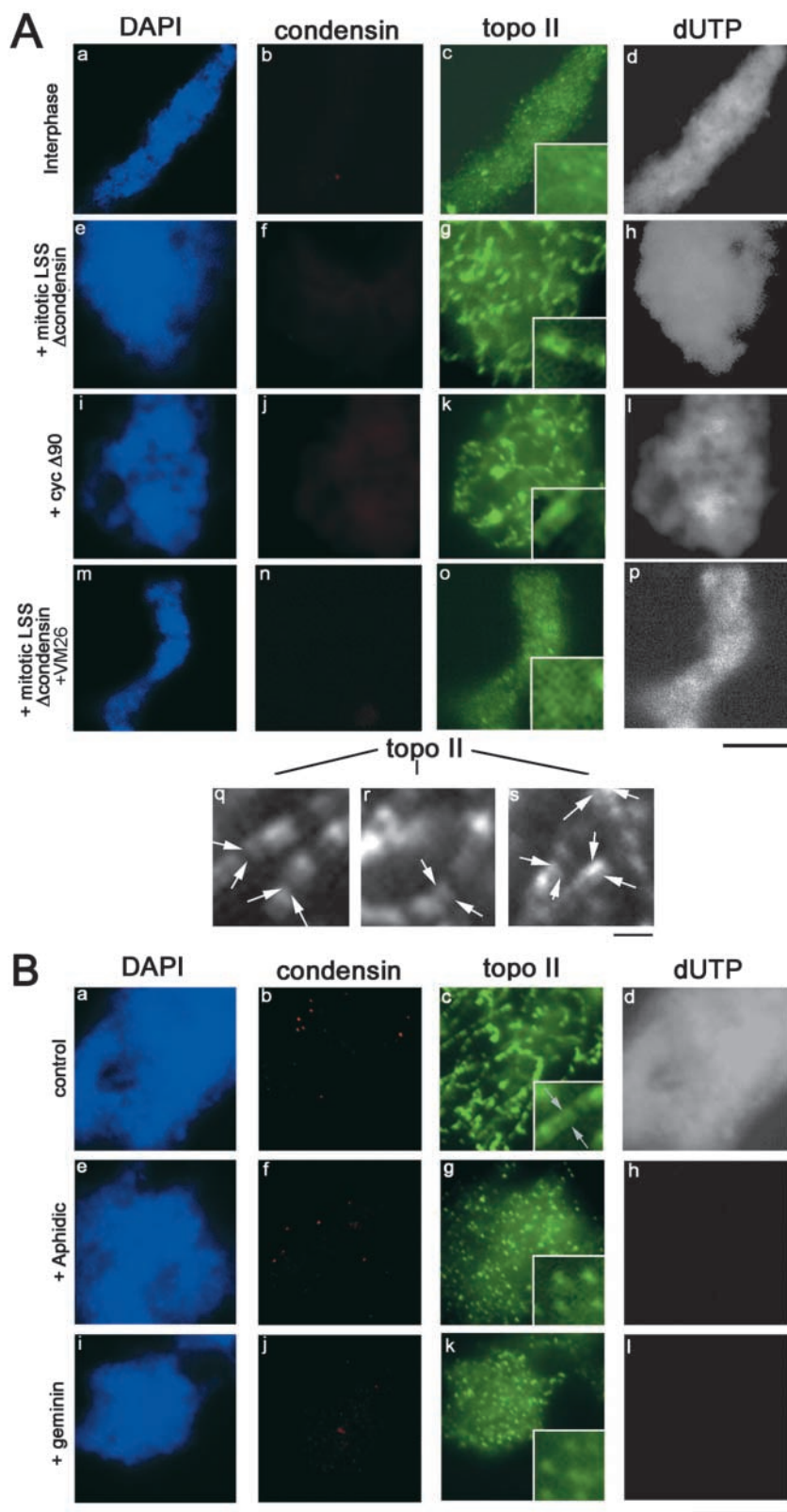


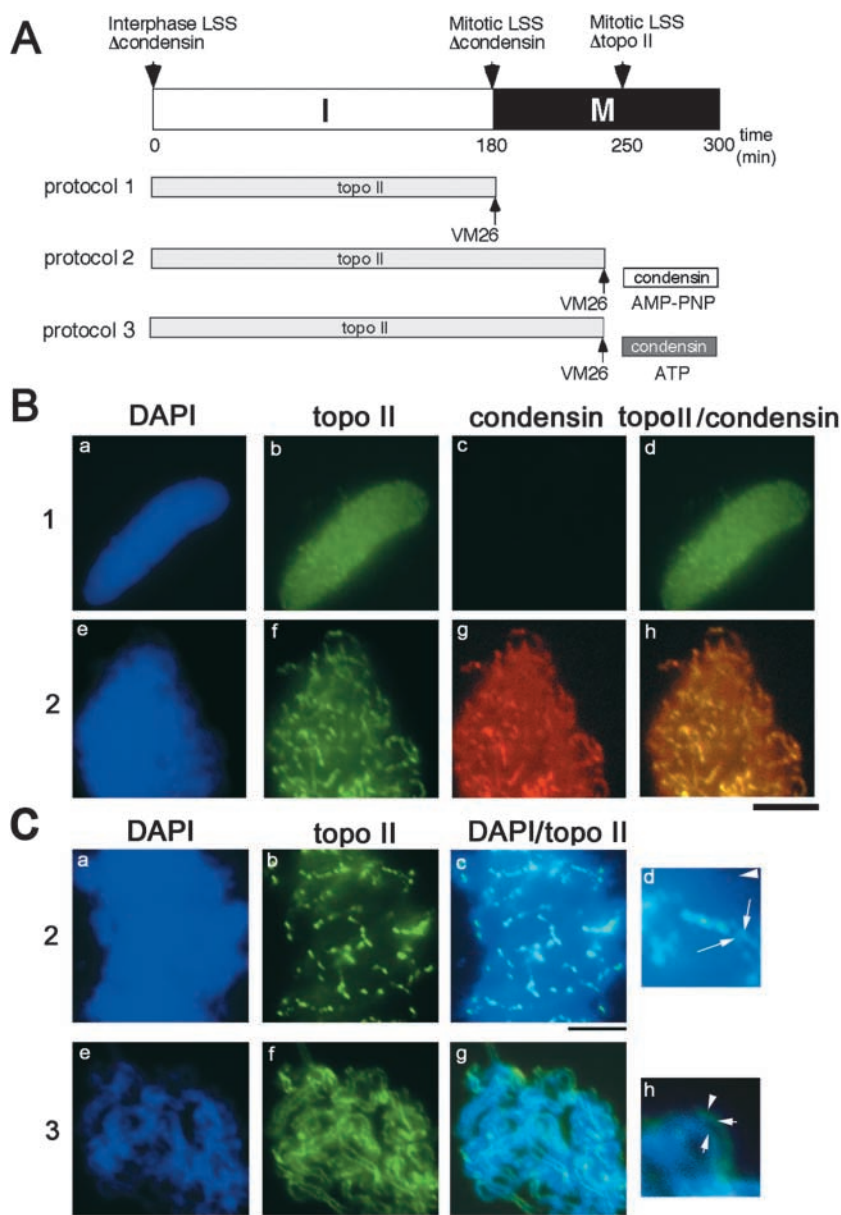
Figure 4. Topo II is concentrated on axial structures in mitosis whose assembly depends on preceding DNA replication. (A) Sperm chromatin was incubated with interphase LSS that had been immunodepleted of condensin. After incubation at 22°C for 180 min, interphase chromatin (a–d) was converted into mitosis by adding either condensin-depleted mitotic LSS (e–h and m–p) or purified recombinant cyclin B Δ 90 (cyc Δ 90; i–l). When required, VM-26 was added before mitotic entry (m–p). Staining was done with anti-condensin (XCAP-G; b, f, j, and n) and anti-topo II (c, g, k, and o) antibodies. DNA was counterstained with DAPI (a, e, i, and m), and DNA replication was assayed with incorporation of biotinylated dUTP (d, h, l, and p). Insets in the third column represent higher magnification of the topo II signals. Bar, 10 μ m (3.3 μ m for the insets). More examples of topo II-containing axes are also shown below (q–s). The arrows show two parallel axes stained with anti-topo II. Bar, 1 μ m. (B) Sperm chromatin was incubated with interphase LSS that had been immunodepleted of condensin in the presence of aphidicolin (e–h), geminin (i–l), or neither of them (a–d). After incubation for 180 min, cyclin B Δ 90 was added to induce mitosis. The assembled structures were stained as in A. Insets in the third column represent higher magnification of the topo II signals. The arrows show two parallel axes stained by topo II. Bar, 10 μ m (3.3 μ m for the insets).

not identical, structures were formed (Fig. 3 B, e–h). This was an unexpected observation because chromosomes failed to be assembled when HSS was used in the same protocol (Fig. 2 B, e–g). Co-immunostaining experiments showed that both condensin and topo II bound to these chromosomes (Fig. 3 B, f and g) as efficiently as those as-

sembled in the absence of VM-26 (Fig. 3 B, b and c). Addition of VM-26 before the onset of mitosis (Fig. 3 A, protocol 3) prevented the formation of chromosomes (Fig. 3 B, i–l). Thus, topo II must be active for a certain period in mitosis before chromosomes are assembled by subsequent action of condensin.

Figure 5. **Topo II-containing axes can be converted by condensin into chromosomes.**

(A) Experimental scheme. Sperm chromatin was incubated with interphase that had been immunodepleted of condensin. After incubation at 22°C for 180 min (protocol 1), interphase chromatin (a–d) was converted into mitosis by adding condensin-depleted mitotic LSS. After another 60 min (at 240 min), the mixture was split into two aliquots; one was treated with 10 μ M VM-26 and 1 mM AMP-PNP (protocol 2), and the other was treated with 10 μ M VM-26 with 1 mM ATP (protocol 3). At 250 min, topo II-depleted mitotic LSS was added to allow condensin to function. (B) Interphase chromatin was either fixed at 180 min (a–d) or converted to mitosis (e–h) after protocol 1 or 2, respectively. After chromosomes were assembled, staining was done with anti-topo II (b and f) and anti-condensin (XCAP-G; c and g). The merged images of topo II and condensin are also shown (d and h). DNA was counterstained with DAPI (a and e). Bar, 10 μ m. (C) Chromosomes were assembled after protocol 2 (a–d) or 3 (e–h), and staining was done with anti-topo II (b and f). DNA was counterstained with DAPI (a and e). The merged images of topo II and DNA are also shown (c and g). Insets (d and h) represent higher magnification of the topo II-containing axes (arrows) and DNA region (arrowheads). Bar, 10 μ m (for a–c and e–g); 2 μ m (for d and h).



DNA replication primes the assembly of topo II-containing axes that can be visualized on mitotic entry

The different observations made in HSS and LSS suggested that the temporal requirement for topo II in mitotic chromosome assembly may be affected by preceding DNA replication. As an attempt to test this possibility, we first determined the localization of topo II on chromatin in LSS, after DNA replication but before the addition of condensin. Sperm chromatin was first incubated with condensin-depleted, interphase LSS to allow DNA replication. At this stage, topo II was distributed uniformly on chromatin (Fig. 4 A, a–d). The extract was then converted into mitosis by adding mitotic LSS that had been depleted of condensin. Although no individual chromosomes were formed under this condition (Fig. 4 A, e), a subpopulation of topo II became concentrated on multiple axis-like structures within a chromatin mass (Fig. 4 A, g). The topo II staining along the axes was irregular in intensity, and was often disrupted as the axes

would come out of the focal plane (Fig. 4 A, g inset and q–s), suggesting that these structures may not be continuous ones. Similar structures were observed when mitotic entry was triggered by the addition of purified recombinant cyclin B Δ 90 (Fig. 4 A, k). We also noticed that each of the axial structures containing topo II was often composed of two distinct lines (Fig. 4 A, g and k, inset; q–s, arrows). The addition of VM-26 on mitotic entry suppressed the formation of axial structure (Fig. 4 A, m–p), suggesting that topo II activity is required during mitosis for this process. Because the treatment with VM-26 decreases the mobility of topo II in vivo (Christensen et al., 2002), it is equally possible that a high turnover of topo II is important for the formation of this structure.

To test whether the formation of the axial structures in mitosis may require preceding DNA replication, we added aphidicolin, a specific inhibitor of DNA polymerase α , to the interphase extract. Efficient blocking of DNA replication was

confirmed by the lack of dUTP incorporation into chromatin (Fig. 4 B, h). We found that this treatment abolished the formation of topo II-containing axes, instead producing a punctuate distribution of topo II signals throughout chromatin (Fig. 4 B, g). Similar observations were made when aphidicolin was replaced with geminin, a protein factor that inhibits the initiation of DNA replication by binding directly to the replication factor Cdt1 (Fig. 4 B, i–l; Tada et al., 2001). Thus, DNA replication is a prerequisite of the formation of the topo II-containing subchromosomal structures.

Active condensin converts the topo II-containing axes into chromosome-like structures

We reasoned that the topo II-containing axes might function as templates for the subsequent action of condensin in LSS. To investigate the functional interaction between topo II and condensin more closely, we took advantage of the use of AMP-PNP, a nonhydrolyzable analogue of ATP that allows condensin to bind to DNA, but prevents its DNA supercoiling/knotting activity in vitro (Kimura and Hirano, 1997; Kimura et al., 1999; Bazzett-Jones et al., 2002). The standard chromosome assembly protocol was used in LSS, except that AMP-PNP was added together with condensin at the final step (Fig. 5 A, protocol 2). The diffuse localization of topo II in interphase chromatin (Fig. 5 B b, protocol 1) was converted into axial structures on entry into mitosis. The topo II-containing structure remained intact after addition of VM-26 and condensin in the presence of AMP-PNP (Fig. 5 B, f). Under this condition, no individual chromosomes were assembled (Fig. 5 B, e), most likely because AMP-PNP inhibited the action of condensin. Interestingly, condensin was found to largely colocalize with topo II within the uncondensed chromatin (Fig. 5 B, g and h), suggesting that condensin may have high affinity for the topo II-enriched axes. Close inspection of the structure showed that DNA extended out of the topo II-containing axes in the presence of AMP-PNP (Fig. 5 C, d). When AMP-PNP was replaced with ATP at the final stage of incubation (Fig. 5 A, protocol 3), the fuzzy chromatin mass was converted into well-individualized chromosomes (Fig. 5 C, e). The topo II signal was converted from thin axes to chromosome-like structures (Fig. 5 C, f), and largely overlapped with the DAPI-stained areas (Fig. 5 C, g and h). These results suggest that, once the topo II-containing axes are formed in a DNA replication-dependent manner, subsequent addition of condensin is sufficient to assemble chromosomes in the absence of active topo II.

DNA replication allows topo II to be tightly associated with chromosomes

Topo II activity is required for chromosome assembly in HSS. However, once assembly is completed, topo II is easily extractable from single-chromatid chromosomes with medium salt concentrations without changing their global structure (Hirano and Mitchison, 1993). We wished to test whether replication-dependent assembly of topo II-containing axes might alter salt-extractability of topo II from chromosomes. To this end, metaphase chromosomes with sister chromatids were assembled in LSS (Fig. 6 A, a–d) and then

treated with buffer containing 0.6 M NaCl (Fig. 6 A, e–h). Under this condition, both topo II and condensin tightly associated with chromatin, and the staining for these proteins (Fig. 6 A, b and c) remained unchanged after salt treatment (Fig. 6 A, f and g). In contrast, when chromosomes were assembled in HSS (Fig. 6 A, i–l) and treated in the same way, the topo II signal was largely diminished (Fig. 6 A, n), whereas condensin staining was unchanged (Fig. 6 A, o). Immunoblotting experiments confirmed that chromosome-bound topo II is more resistant to salt extraction in LSS than in HSS (unpublished data). When aphidicolin was added into LSS to prevent DNA replication, topo II became sensitive to salt extraction (Fig. 6 B), suggesting that DNA replication accounts, at least in part, for the difference in topo II association between HSS and LSS.

To test whether the tight association of topo II with the double-chromatid chromosomes may be achieved through its functional interaction with condensin, chromosomes were assembled in LSS in the absence of condensin (Fig. 6 C, a–d) and were then treated with 0.6 M NaCl (Fig. 6 C, e–h). We found that the topo II signal was not changed after salt treatment even under this condition (Fig. 6 C, compare b with f). These results suggest that DNA replication allows topo II to become tightly associated with chromosomes, and that this alteration of topo II behavior occurs independently of condensin.

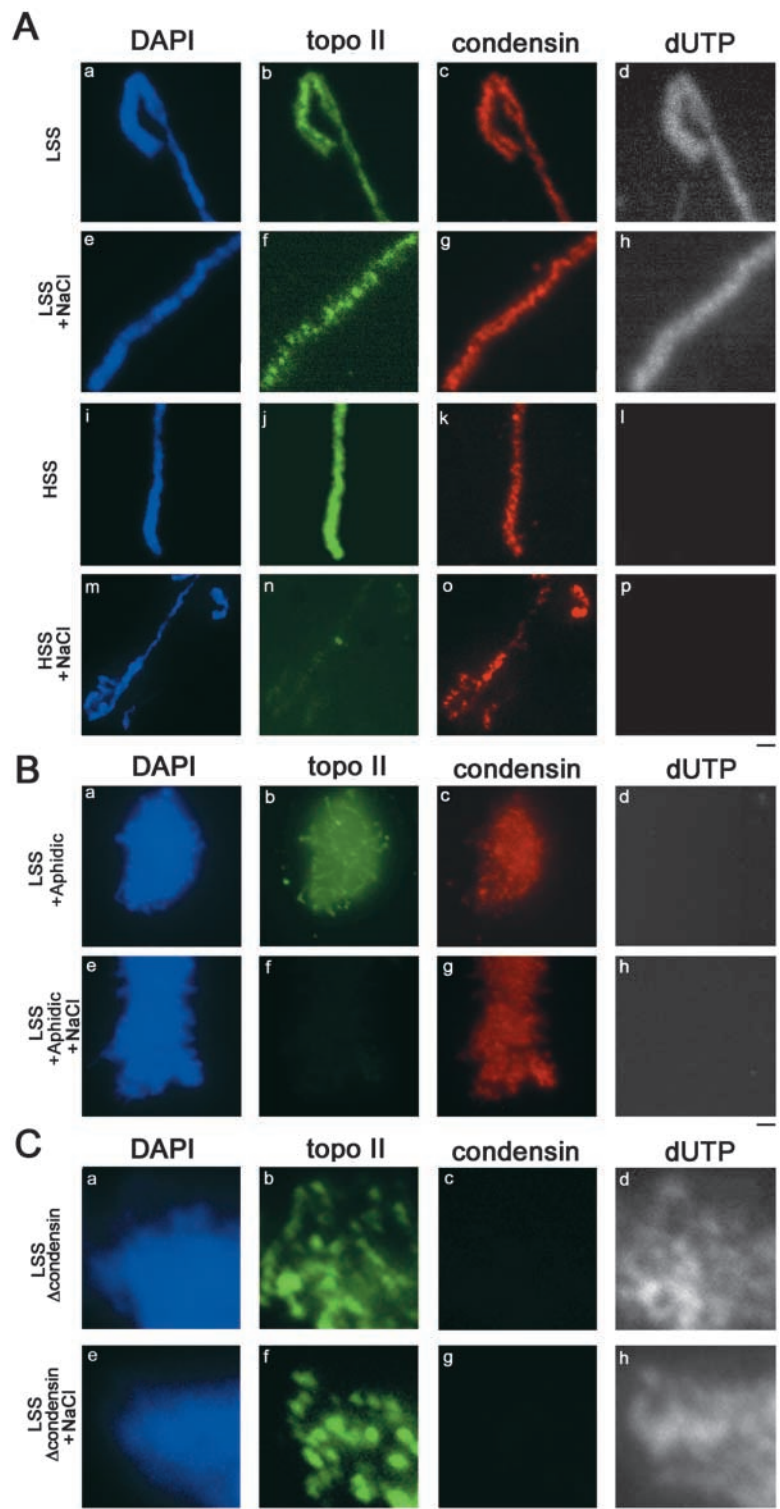
Discussion

Topo II and dynamic organization of chromosomes

The current paper addresses the functional interplay of topo II and condensin in chromosome assembly by using the *Xenopus* egg extracts. Our results show that although topo II and condensin have different roles in chromosome condensation, their actions appear to be tightly linked. In fact, when unreplicated chromatin is directly converted into single-chromatid chromosomes in HSS, the two proteins must be active simultaneously (Fig. 7, right). Interestingly, however, the simultaneous requirement for the two proteins can be circumvented when chromosome assembly is induced after allowing DNA replication in LSS. Under this condition, it becomes possible to detect a subchromosomal structure containing topo II in the absence of condensin. We suggest that chromosome condensation after DNA replication can be dissected into two distinct steps: (1) an early step involving the formation of topo II axis; and (2) a late event involving the condensin-mediated compaction (Fig. 7, left). A requirement for topo II activity before the formation of chromosomes has also been observed in vivo (Downes et al., 1994; Gimenez-Abian et al., 2000).

The molecular nature of the topo II-containing axes remains to be determined. The current analysis does not allow us to be certain whether they are continuous filament-like structures or whether topo II signals nonrandomly distribute along each of the duplicated chromosomes. It is interesting to note that some of the properties associated with the topo II-containing structures reported here are reminiscent of the “chromatid core” of mammalian chromosomes that can be visualized by a silver impregnation method (Gimenez-Abian et al., 1995). This core structure is first detectable in

Figure 6. DNA replication allows topo II to be tightly associated with chromosomes. (A) Sperm chromatin was incubated with interphase LSS, and then half a volume of mitotic LSS was added to assemble double-chromatid chromosomes (a–h). Alternatively, a combination of interphase and mitotic HSS was used to assemble single-chromatid chromosomes without DNA replication (i–p). The assembly mixtures were untreated (a–d and i–l) or treated with 0.6 M extra concentrations of NaCl (e–h and m–p) for 20 min at 22°C, fixed, and stained with anti-topo II (b, f, j, and n), anti-condensin (XCAP-G; c, g, k, and o), and DAPI (a, e, i, and m). DNA replication was assayed with incorporation of biotinylated dUTP (d, h, l, and p). Bar, 1 μ m. (B) Chromosomes were assembled in LSS in the presence of aphidicolin, and the assembly mixtures were untreated (a–d) or treated as above with 0.6 M NaCl (e–h). (C) Chromosomes were assembled in LSS that had been immunodepleted of condensin, and the mixture was untreated (a–d) or treated as above with 0.6 M NaCl (e–h). Staining was performed as described in A. Bar, 1 μ m.



prophase as a single axis, which separates at prometaphase to form two chromatid axes in a topo II–dependent manner.

Previous studies showed that topo II is tightly associated with metaphase chromosomes isolated from somatic cells, leading to the proposal that topo II plays a role in the structural maintenance of chromosomes as an integral component of the scaffold (Earnshaw et al., 1985; Gasser et al., 1986). In striking contrast, topo II was found to be easily extracted from single-chromatid chromosomes assembled in

HSS without changing their overall structure (Hirano and Mitchison, 1993). The current results show that the presence or absence of preceding DNA replication affects the distribution and salt-extractability of topo II on chromosomes, providing at least a partial explanation for the discrepancy between the previous studies. Our conclusion is further supported by the observation that preventing DNA replication with aphidicolin in LSS abolishes the tight association of topo II with the chromosomes. Because topo II

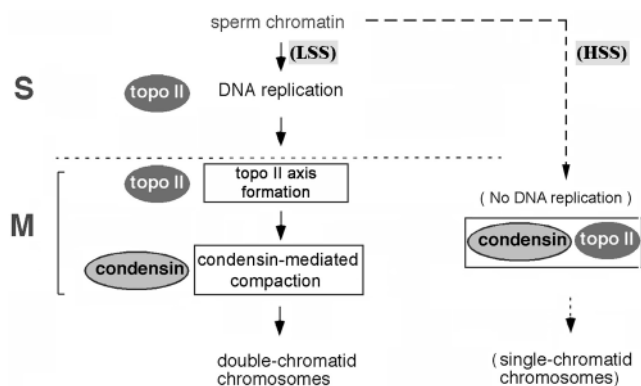


Figure 7. **Chromosome condensation in the presence or absence of DNA replication.** For details, see the text.

displays highly dynamic behavior in vivo during both interphase and mitosis (Swedlow et al., 1993; Christensen et al., 2002; Tavormina et al., 2002), we favor the idea that topo II function as part of a complex molecular assembly that undergoes a dynamic rearrangement during the cell cycle and links interphase chromatin organization to mitotic chromosome architecture. This is not necessarily inconsistent with the model that topo II may play a role in forming chromosome loops by binding to scaffold-attachment regions in an early stage of chromosome assembly (for review see Hart and Laemmli, 1998). However, our current results do not address the question of whether topo II function may continuously be required for the structural maintenance of chromosomes after their assembly is complete.

Topo II links DNA replication to chromosome condensation

Chromosome condensation is not a simple compaction process of a linear DNA molecule, and it requires the relief of a number of physical constraints that would otherwise impede compaction (Hirano, 2000). It has been proposed that the DNA decatenation activity of topo II is required to untangle catenations between different chromosomes (nonreplicative catenanes) and between sister chromatids (replicative catenanes) before the completion of mitosis (Downes et al., 1994; Gimenez-Abian et al., 2000). Although topo II activity is not required for DNA replication, per se, it acts behind the replication fork and unlinks replicating DNA by removing precatenates in *Xenopus* egg extracts (Lucas et al., 2001). This unlinking process does not require entry into mitosis, at least in the case of small circular DNA substrates. It is tempting to speculate that the close association of topo II with the fork movement may prime the assembly of a sub-chromosomal structure during S-phase, which can only be visualized on mitotic entry. Such a replication-dependent reorganization of topo II distribution might also facilitate the removal of nonreplicative catenanes. Our results show that topo II plays a crucial role in linking DNA replication to mitotic chromosome condensation. Several recent studies also start to reveal this previously understated connection between the two important chromosomal events (for review see Pflumm, 2002). For example, several origin recognition complex mutants in *Drosophila* produce shorter and thicker

chromosomes (Pflumm and Botchan, 2001), or irregularly condensed chromosomes with the severest defect in late-replicating regions (Loupard et al., 2000). It has been hypothesized that the correct timing of DNA replication or the density of functional replication origin might affect the assembly of chromosomes during subsequent mitosis. It would be interesting to test whether mutations in replication factors may perturb proper distribution of topo II during DNA replication and thereby lead to condensation defects.

The interplay between topo II and condensin

How do topo II and condensin functionally interact with each other to assemble chromosomes? Unlike topo II, which binds to chromatin throughout the cell cycle, the binding of condensin to chromosomes is mitosis-specific in the cell-free extracts (Hirano et al., 1997; Losada et al., 1998). Our current data clearly show that topo II and condensin are both required for chromosome assembly in the presence or absence of DNA replication. However, after DNA replication, topo II-containing axial structure is observed in the absence of condensin, and this structure can be converted into a metaphase chromosome-like structure by subsequent addition of condensin. One potential mechanism might be that the targeted condensin drives DNA supercoiling, which in turn facilitates topo II-mediated decatenation of the nonreplicative and replicative catenanes, and thereby allows compaction of each chromatid. It should be noted that the actions of the two proteins in chromosome assembly are tightly linked under normal conditions. The topo II-containing axes reported in the current paper would not be visualized when condensin is present.

A number of genetic studies from different model organisms clearly show that condensin function is required for proper chromosome condensation and segregation in vivo (Saka et al., 1994; Strunnikov et al., 1995; Sutani et al., 1999; Ouspenski et al., 2000; Bhalla et al., 2002; Hagstrom et al., 2002). However, it remains to be determined exactly how condensin contributes to the assembly of metaphase chromosomes at a mechanistic level. For example, the *Drosophila* SMC4 mutants produce very fat chromosomes with unresolved sister chromatids, suggesting that condensin function may be required for sister chromatid resolution, but not for axial compaction (Steffensen et al., 2001). When SMC-4 is depleted by RNA interference in *Caenorhabditis elegans* embryos, the most drastic condensation defect is observed in prometaphase, but not in metaphase (as judged by metaphase plate formation; Hagstrom et al., 2002; Kaitna et al., 2002). Additional factors, including topo II, are likely to contribute to a certain level of compaction. It would be informative to carefully compare and contrast the defective phenotypes associated with condensin and topo II mutants in different organisms. It would also be important to note that the relative contribution of condensin and other factors to chromosome architecture might vary depending on the size of chromosomes, assembly pathways (with or without preceding DNA replication), or developmental stages. For instance, in organisms that contain short chromosomes, like *S. cerevisiae*, chromosome compaction requires condensin, but apparently not topo II (Lavoie et al., 2002). Future studies should integrate information from biochemistry, genet-

ics, and structural analysis of chromosomes to fully understand the interplay of condensin and topo II in chromosome assembly at a mechanistic level.

Materials and methods

Preparation of *Xenopus* egg extracts

Mitotic HSS were prepared with the procedure described by Hirano et al. (1997). Interphase and mitotic LSS were prepared as described previously (Losada et al., 1998), with minor modifications. After low speed centrifugation, LSS were added dropwise (20 μ l) into liquid nitrogen to optimize freezing, and the beads were collected and stored at -70°C (Blow and Laskey, 1986).

Immunodepletions

Immunodepletions of topo II and condensin were performed as described previously (Hirano and Mitchison, 1993; Hirano et al., 1997), with minor modifications. For condensin depletion, a mixture of affinity-purified anti-XCAP-C, -E, and -G (6 μ g each) and anti-XCAP-D2 (12 μ g) was incubated with 30 μ l Affi-Prep[®] Protein A support (Bio-Rad Laboratories) for 1 h. For topo II depletion, 30 μ l of anti-topo II serum was loaded onto 30 μ l beads. For mock depletion, 30 μ g control IgG or 30 μ l nonimmune rabbit antiserum was used. After washing the antibody-coupled beads with XBE2 (10 mM K-Hepes, pH 7.7, 100 mM KCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 5 mM EGTA, and 50 mM sucrose), 100 μ l HSS or LSS supplemented with an ATP-regenerating system (Hirano and Mitchison, 1993) was added and incubated on ice for 1 h with occasional mixing. The supernatants were recovered by two rounds of brief spins and used as freshly depleted extracts. For immunodepletion of topo II, two successive rounds of incubation were performed.

Chromosome assembly in *Xenopus* egg extracts

Chromosomes were assembled from sperm chromatin in HSS or LSS as described previously (Hirano et al., 1997; Losada et al., 1998), except that topo II- or condensin-depleted extracts were used as indicated. To monitor the efficiency of DNA replication, biotin-16-dUTP (Boehringer) was added into interphase extracts at a final concentration of 10 μ M. For DNA replication inhibition, 100 μ g/ml aphidicolin or 1.5 μ g/ml of nondegradable recombinant geminin (McGarry and Kirschner, 1998) was added to the extracts. Mitosis was induced by adding 1/2 volume of mitotic extracts or purified recombinant cyclin B490 (Solomon et al., 1990). When necessary, VM-26 (4'-demethylepipodophyllotoxin thenylidene- β -D-glucoside; a gift from Bristol-Myers Squibb Company) was added at a final concentration of 10 μ M. AMP-PNP or ATP was added to 1 mM when indicated.

Analyses of chromosomes assembled in vitro

Chromosome assembly reactions were performed as described previously (Hirano et al., 1997; Losada et al., 1998), by incubating sperm chromatin with HSS or LSS at 22°C for 2 h. Salt extraction of chromosomes was done as described previously (Hirano and Mitchison, 1993), by adding 1/10 reaction volume of buffer containing 10 \times NaCl into assembly reactions. For biochemical analysis of chromosomal proteins (Fig. 1 A), the reaction mixtures were loaded onto a 30% sucrose cushion and spun at 10,000 rpm for 15 min (Hirano and Mitchison, 1994). For morphological analysis (Figs. 1–6), the assembled chromosomes were fixed and recovered onto coverslips by centrifugation through a 30% glycerol cushion, as described previously (Hirano and Mitchison, 1991), with minor modifications. 10 μ l assembly mixture was fixed by the addition of 50 μ l 0.8% formaldehyde and incubated at RT for 10 min. For sedimentation of salt-treated chromosomes or chromatin assembled in condensin-depleted extracts, a 10% (instead of 30%) glycerol cushion was used. The coverslips were washed with TBS containing 0.1% Triton X-100 (TBST) for 10 min, and were blocked with TBS containing 2% BSA (TBS-BSA) at 22°C for 1 h in a humid chamber. Samples were then incubated with primary antibodies diluted in TBS-BSA at 4°C overnight. The antibodies used were anti-topo II (Hirano and Mitchison, 1993), anti-cohesin (XSMC3; Losada et al., 1998), and anti-condensin (Hirano et al., 1997). For double immunostaining, a combination of mouse anti-condensin (XCAP-G) and rabbit anti-topo II antibodies was used. After washing with TBST, fluorescein- or rhodamine-labeled goat anti-rabbit IgG or donkey anti-mouse antibodies (Jackson ImmunoResearch Laboratories) were applied at 1:100 dilution for 1 h at 22°C , followed by TBST washes and incubation with 10 μ g/ml fluorescein-conjugated avidin D for 30 min. After washing with TBST and DAPI counterstaining, samples were mounted in medium (Vectashield[®]) for microscopy.

Other assays

Topo II-mediated DNA cleavage and kinetoplast DNA decatenation assays were done as described previously (Hirano and Mitchison, 1991, 1993).

We thank Ana Losada and Takao Ono for critically reading the manuscript and Peter Gillespie (Cold Spring Laboratory) for his gift of recombinant geminin and for technical advice for preparation of LSS.

This work was supported by a grant from the National Institutes of Health (to T. Hirano) and by fellowships from the Cold Spring Harbor Laboratory Association and the Human Frontier Science Program (to O. Cuvier).

Submitted: 5 September 2002

Revised: 15 January 2003

Accepted: 15 January 2003

References

- Adachi, Y., M. Luke, and U.K. Laemmli. 1991. Chromosome assembly in vitro: topoisomerase II is required for condensation. *Cell* 64:137–148.
- Anderson, D.E., A. Losada, H.P. Erickson, and T. Hirano. 2002. Condensin and cohesin display different arm conformations with characteristic hinge angles. *J. Cell Biol.* 156:419–424.
- Bazzett-Jones, D.P., K. Kimura, and T. Hirano. 2002. Efficient supercoiling of DNA by a single condensin complex as revealed by electron spectroscopic imaging. *Mol. Cell* 9:1183–1190.
- Bhalla, N., S. Biggins, and A.W. Murray. 2002. Mutation of YCS4, a budding yeast condensin subunit, affects mitotic and nonmitotic chromosome behavior. *Mol. Biol. Cell* 13:632–645.
- Bhat, M.A., A.V. Philp, D.M. Glover, and H.J. Bellen. 1996. Chromatid segregation at anaphase requires the barren product, a novel chromosome associated protein that interacts with topoisomerase II. *Cell* 87:1103–1114.
- Blow, J.J., and R.A. Laskey. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell* 47:577–587.
- Christensen, M.O., M.K. Larsen, H.U. Barthelmes, R. Hock, C.L. Andersen, E. Kjeldsen, B.R. Knudsen, O. Westergaard, F. Boege, and C. Mielke. 2002. Dynamics of human DNA topoisomerases II α and II β in living cells. *J. Cell Biol.* 157:31–44.
- Cobbe, N., and M.M.S. Heck. 2000. SMCs in the world of chromosome biology—from prokaryotes to higher eukaryotes. *J. Struct. Biol.* 129:123–143.
- Downes, C.S., D.J. Clarke, A.M. Mullinger, J.F. Gimenez-Abian, A.M. Creighton, and R.T. Johnson. 1994. A topoisomerase II-dependent G2 cycle checkpoint in mammalian cells. *Nature* 372:467–470.
- Earnshaw, W.C., B. Halligan, C.A. Cooke, M.M.S. Heck, and L.F. Liu. 1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* 100:1706–1715.
- Gasser, S.M., T. Laroche, J. Falquet, E. Boy de la Tour, and U.K. Laemmli. 1986. Metaphase chromosome structure. Involvement of topoisomerase II. *J. Mol. Biol.* 188:613–629.
- Gimenez-Abian, J.F., D.J. Clarke, A.M. Mullinger, C.S. Downes, and R.T. Johnson. 1995. A postprophase topoisomerase II-dependent chromatid core separation step in the formation of metaphase chromosomes. *J. Cell Biol.* 131:7–17.
- Gimenez-Abian, J.F., D.J. Clarke, J. Devlin, M.I. Gimenez-Abian, C. De la Torre, R.T. Johnson, A.M. Mullinger, and C.S. Downes. 2000. Premitotic chromosome individualization in mammalian cells depends on topoisomerase II activity. *Chromosoma* 109:235–244.
- Hagstrom, K.A., V.F. Holmes, N.R. Cozzarelli, and B.J. Meyer. 2002. *C. elegans* condensin promotes mitotic chromosome architecture, centromere organization, and sister chromatid segregation during mitosis and meiosis. *Genes Dev.* 16:729–742.
- Hart, C.M., and U.K. Laemmli. 1998. Facilitation of chromatin dynamics by SARs. *Curr. Opin. Genet. Dev.* 8:519–525.
- Hirano, T. 2000. Chromosome cohesion, condensation and separation. *Annu. Rev. Biochem.* 69:115–144.
- Hirano, T. 2002. The ABCs of SMC proteins: two-armed ATPases for chromosome condensation, cohesion, and repair. *Genes Dev.* 16:399–414.
- Hirano, T., and T.J. Mitchison. 1991. Cell cycle control of higher-order chromatin assembly around naked DNA in vitro. *J. Cell Biol.* 115:1479–1489.
- Hirano, T., and T.J. Mitchison. 1993. Topoisomerase II does not play a scaffolding role in the organization of mitotic chromosomes assembled in *Xenopus* egg extracts. *J. Cell Biol.* 120:601–612.
- Hirano, T., and T.J. Mitchison. 1994. A heterodimeric coiled-coil protein required

- for mitotic chromosome condensation in vitro. *Cell*. 79:449–458.
- Hirano, T., R. Kobayashi, and M. Hirano. 1997. Condensin, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a *Xenopus* homolog of the *Drosophila* Barren protein. *Cell*. 89:511–521.
- Holmes, V.F., and N.R. Cozzarelli. 2000. Closing the ring: links between SMC proteins and the partitioning, condensation and supercoiling of chromosomes. *Proc. Natl. Acad. Sci. USA*. 97:1322–1324.
- Kaitna, S., P. Pasierbek, M. Jantsch, J. Loidl, and M. Glotzer. 2002. The aurora B kinase AIR-2 regulates kinetochores during mitosis and is required for separation of homologous chromosomes during meiosis. *Curr. Biol.* 12:798–812.
- Kimura, K., and T. Hirano. 1997. ATP-dependent positive supercoiling of DNA by 13S condensin: a biochemical implication for chromosome condensation. *Cell*. 90:625–634.
- Kimura, K., and T. Hirano. 2000. Dual roles of the 11S regulatory subcomplex in condensin functions. *Proc. Natl. Acad. Sci. USA*. 97:11972–11977.
- Kimura, K., V.V. Rybenkov, N.J. Crisona, T. Hirano, and N.R. Cozzarelli. 1999. 13S condensin actively reconfigures DNA by introducing global positive writhe: implications for chromosome condensation. *Cell*. 98:239–248.
- Kimura, K., O. Cuvier, and T. Hirano. 2001. Chromosome condensation by a human condensin complex in *Xenopus* egg extracts. *J. Biol. Chem.* 276:5417–5420.
- Koshland, D., and A. Strunnikov. 1996. Mitotic chromosome condensation. *Annu. Rev. Cell Dev. Biol.* 12:305–333.
- Lavoie, B.D., K.M. Tuffo, S. Oh, D. Koshland, and C. Holm. 2000. Mitotic chromosome condensation requires Brn1p, the yeast homologue of Barren. *Mol. Biol. Cell*. 11:1293–1304.
- Lavoie, B.D., E. Hogan, and D. Koshland. 2002. In vivo dissection of the chromosome condensation machinery: reversibility of condensation distinguishes contributions of condensin and cohesin. *J. Cell Biol.* 156:805–815.
- Losada, A., M. Hirano, and T. Hirano. 1998. Identification of *Xenopus* SMC protein complexes required for sister chromatid cohesion. *Genes Dev.* 12:1986–1997.
- Loupart, M.L., S.A. Krause, and M.S. Heck. 2000. Aberrant replication timing induces defective chromosome condensation in *Drosophila* ORC2 mutants. *Curr. Biol.* 10:1547–1556.
- Lucas, L., T. Germe, M. Chevrier-Miller, and O. Hyrien. 2001. Topoisomerase II can unlink replicating DNA by precatenane removal. *EMBO J.* 20:6509–6519.
- McGarry, T.J., and M.W. Kirschner. 1998. Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell*. 93:1043–1053.
- Murray, A.W. 1991. Cell cycle extracts. *Methods Cell Biol.* 36:581–605.
- Ouspenski, I.I., O.A. Cabello, and B.R. Brinkley. 2000. Chromosome condensation factor Brn1p is required for chromatid separation in mitosis. *Mol. Biol. Cell*. 11:1305–1313.
- Pflumm, M.F. 2002. The role of DNA replication in chromosome condensation. *Bioessays*. 24:411–418.
- Pflumm, M.F., and M.R. Botchan. 2001. Orc mutants arrest in metaphase with abnormally condensed chromosomes. *Development*. 128:1697–1707.
- Saka, Y., T. Sutani, Y. Yamashita, S. Saitoh, M. Takeuchi, Y. Nakaseko, and M. Yanagida. 1994. Fission yeast cut3 and cut14, members of a ubiquitous protein family, are required for chromosome condensation and segregation in mitosis. *EMBO J.* 13:4938–4952.
- Solomon, M.J., M. Glotzer, T.H. Lee, M. Philippe, and M.W. Kirschner. 1990. Cyclin activation of p34cdc2. *Cell*. 63:1013–1024.
- Steffensen, S., P.A. Coelho, N. Cobbe, S. Vass, M. Costa, B. Hassan, S.N. Prokopenko, H. Bellen, M.M.S. Heck, and C.E. Sunkel. 2001. A role for *Drosophila* SMC4 in the resolution of sister chromatids in mitosis. *Curr. Biol.* 11:295–307.
- Strunnikov, A.V., E. Hogan, and D. Koshland. 1995. SMC2, a *Saccharomyces cerevisiae* gene essential for chromosome segregation and condensation, defines a subgroup within the SMC family. *Genes Dev.* 9:587–599.
- Sutani, T., T. Yuasa, T. Tomonaga, N. Dohmae, K. Takio, and M. Yanagida. 1999. Fission yeast condensin complex: essential roles of non-SMC subunits for condensation and Cdc2 phosphorylation of Cut3/SMC4. *Genes Dev.* 13:2271–2283.
- Swedlow, J.R., J.W. Sedat, and D.A. Agard. 1993. Multiple populations of topoisomerase II detected in vivo by time-lapse, three-dimensional wide-field microscopy. *Cell*. 73:97–108.
- Tada, S., A. Li, D. Maiorano, M. Mechali, and J.J. Blow. 2001. Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nat. Cell Biol.* 3:107–113.
- Tavormina, P.A., M.G. Come, J.R. Hudson, Y.Y. Mo, W.T. Beck, and G.J. Gorbisky. 2002. Rapid exchange of mammalian topoisomerase II α at kinetochores and chromosome arms in mitosis. *J. Cell Biol.* 158:23–29.
- Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, and M. Yanagida. 1987. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. *Cell*. 50:917–925.
- Yoshimura, S.H., K. Hizume, A. Murakami, T. Sutani, K. Takeyasu, and M. Yanagida. 2002. Condensin architecture and interaction with DNA: regulatory non-SMC subunits bind to the head of SMC heterodimer. *Curr. Biol.* 12:508–513.