MOLECULAR AND CELLULAR BIOLOGY, July 1997, p. 3520–3526 Vol. 17, No. 7
0270-7306/97/$04.00+0

Topoisomerase Function during Replication-Independent Chromatin Assembly in Yeast

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Received 30 December 1996/Returned for modification 24 February 1997/Accepted 10 April 1997

DNA topoisomerases I and II are the two major nuclear enzymes capable of relieving torsional strain in DNA. Of these enzymes, topoisomerase I plays the dominant role in relieving torsional strain during chromatin assembly in cell extracts from oocytes, eggs, and early embryos. We tested if the topoisomerases are used differentially during chromatin assembly in *Saccharomyces cerevisiae* by a combined biochemical and pharmacological approach. As measured by plasmid supercoiling, nucleosome deposition is severely impaired in assembly extracts from a yeast mutant with no topoisomerase I and a temperature-sensitive form of topoisomerase II (strain top1-top2). Expression of wild-type topoisomerase II in strain top1-top2 fully restored assembly-driven supercoiling, and assembly was equally efficient in extracts from strains expressing either topoisomerase I or II alone. Supercoiling in top1-top2 extract was rescued by adding back either purified topoisomerase I or II. Using the topoisomerase II poison VP-16, we show that topoisomerase II activity during chromatin assembly is the same in the presence and absence of topoisomerase I. We conclude that both topoisomerases I and II can provide the DNA relaxation activity required for efficient chromatin assembly in mitotically cycling yeast cells.

The DNA topoisomerases serve important functions in the transmission and expression of genetic information, functions that largely depend on the ability of these enzymes to cleave and religate duplex DNA molecules in a concerted reaction that can convert supercoiled DNA to relaxed closed circular DNA (reviewed in references 29 and 30). In the nucleus, most of the topoisomerase or nicking-closing activity is provided by two topoisomerases, DNA topoisomerases I and II. Topoisomerases I and II relax DNA during such essential cellular processes as replication and transcription, when chain elongation generates torsional strain that must be relieved in order for a polymerase to move rapidly along its template (reviewed in references 8 and 29 to 31). Besides replication and transcription, chromatin assembly is also promoted by the relaxation of DNA. Relaxation during chromatin assembly is favored because DNA is twisted when it wraps around a histone octamer to form a nucleosome (reviewed in references 27 and 32), and this twisted conformation is energetically unstable (21). Theoretically, DNA relaxation during chromatin assembly could be provided by separate nicking and closing enzymes or by a topoisomerase. Biochemical and pharmacological approaches have been exploited to identify the activity that relaxes DNA during chromatin assembly in biological nucleosome reconstitution systems. In extracts of *Xenopus* oocytes and eggs (1) and *Drosophila* embryos (4), DNA relaxation during chromatin assembly is largely performed by topoisomerase I (reviewed in reference 2; see also commentaries in references 9 and 15). This use of topoisomerase I in preference to topoisomerase II was also observed when chromatin assembly was examined by microinjecting plasmids into oocyte nuclei (1). The functional dominance of topoisomerase I over topoisomerase II during chromatin assembly is unusual, since these enzymes act redundantly to relax torsional strain generated during transcription and replication (reviewed in references 8 and 29 to 31). We therefore initiated a study to examine the molecular basis for the preferred use of topoisomerase I over topoisomerase II during chromatin assembly.

We performed our experiments in a chromatin assembly extract from the budding yeast *Saccharomyces cerevisiae* (24), taking advantage of the ability to manipulate the activity of the topoisomerases in yeast by genetic and pharmacological methods. As in oocytes and eggs, we find that a topoisomerase rather than independent nicking and closing activities is required for chromatin assembly. Contrary to our expectations, however, we observed that topoisomerases I and II can function with equal efficiency during chromatin assembly in a yeast extract, and we provide evidence that topoisomerase II is fully active during chromatin assembly in the presence of topoisomerase I. Topoisomerases I and II are redundant in terms of their ability to provide the relaxation activity required for chain elongation by the DNA and RNA polymerases. Our results establish that this functional redundancy extends to another essential cellular process, chromatin assembly.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* TOP$^+$ (same as W303-1a, originally from R. Rothstein, Columbia University; *MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1*), top1 (W303-1a *top1-8*::*LEU2*), top2 (*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 top2-1*), and top1-top2 (RS191 *top1-8*::*LEU2*) were kindly provided by Rolf Sternglanz (5). A364A (13) was from the Yeast Genetic Stock Center. These strains were grown in YEPD at 25°C and harvested when the culture reached late log phase (optical density at 600 nm [OD_{600}] of 3 to 3.5). In some cases, the culture was shifted to 37°C for 1 h prior to harvesting (see figure legends). Strain top1-top2/pTOP2 was obtained by transforming the top1-top2 strain with plasmid YEpTOP2-PGAL1 (33) (gift from James Wang). This plasmid was designed for the overexpression of yeast topoisomerase II under the control of the inducible *GAL1* promoter. Strain top1-top2/pTOP2 was grown in minimal medium (no uracil) supplemented with 2% (wt/vol) glucose. When the cells had reached an OD_{600} of 3, the culture was split in two. One aliquot of cells was left in glucose medium. The other aliquot was spun down, and the cells were then washed with water and resuspended in minimal medium (no uracil) supplemented with 2% (wt/vol) galactose. Both aliquots were further incubated for 4 h at room temperature and then shifted to 37°C for 1 h prior to harvesting.

Extract preparation and chromatin assembly reactions. Chromatin assembly extracts were prepared from frozen cells (23) after grinding in a coffee mill, and nucleosome assembly was monitored by plasmid supercoiling assay to be described elsewhere (24). Reactions were performed with 100 μg of yeast extract, unless otherwise stated, in a final volume of 20 μl for 1 h at 37°C. The template

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for supercoiling reactions was pBluescript SK (Stratagene) internally labeled at the *Hin*dIII site. For the in vitro add-back experiments, we used calf thymus topoisomerase I (15 U/μl; Life Technologies) and human topoisomerase II (2 U/μl; TopoGEN, Inc.). *Escherichia coli* T4 DNA ligase (1 U/μl; Life Technologies) was used for the ligase experiment. Quantitation of supercoiling gels was performed by phosphorimager analysis, using a Fujix Bas 1000 bioimaging analyzer running MacBAS software.

Micrococcal nuclease analysis. The protocol of Schultz et al. (24) was used, with the following modifications. Cells were grown in parallel to stationary phase (OD_{600} of 10). Assembly reaction mixtures (100 μl; 750 μg of protein) received 6 μl of 0.1 M $CaCl_2$ and 4.4 μl of micrococcal nuclease (0.5 U/μl; Sigma). The amount of micrococcal nuclease was reduced by sixfold for the digestions of naked DNA. The products were resolved by 1.5% agarose gel electrophoresis in Tris-glycine running buffer and detected by Southern blotting. In one set of experiments, 500 μg of poly-L-glutamic acid (molecular weight, 15,000 to 50,000; ICN) per ml was included in the assembly reactions to inhibit an activity that appears to destabilize chromatin in our extract and cause poor resolution of higher-order nucleosomal arrays (26). Under the conditions used for assembly with yeast extract (see above), poly-L-glutamate in combination with purified yeast histones and topoisomerase I is unable to assemble nucleosomes, as judged by micrococcal nuclease digestion analysis (11).

Drug inhibition studies. Chromatin assembly reactions were performed as described above, in the presence of increasing concentrations of VP-16 (lot no. C8F14A; Bristol-Myers Squibb). The drug was provided as a 34 mM solution in 20 mg of benzyl alcohol per ml and 30.5% ethanol (as well as some minor salts). Each 20-μl assembly reaction mixture received 1.2 μl of solvent (20 mg of benzyl alcohol per ml, 30.5% ethanol) containing the amount of VP-16 to give the final VP-16 concentrations indicated in Fig. 7. The reaction components were mixed on ice, VP-16 being added last. The reactions were stopped by adding 5 μl of 2.5 g of Sarkosyl per 100 ml–100 mM EDTA, and the resulting mixture was then treated with 1 mg of proteinase K per ml for 30 min at 37°C; 200 μl of 0.3 M sodium acetate–0.5% sodium dodecyl sulfate–10 mM EDTA was then added, the protein was extracted, and DNA was precipitated with ethanol. The products were run on a 0.8% agarose gel in Tris-acetate-EDTA running buffer.

RESULTS

Chromatin assembly in extracts that lack topoisomerase activity. For these experiments, we used a simple whole-cell extract of yeast (23, 25) that supports chromatin assembly (24). Our experiments exploited a combined biochemical and genetic approach in which chromatin assembly extracts were prepared from previously described mutants of DNA topoisomerases I and II (5). We first determined if any activity besides a major DNA topoisomerase could provide the nicking-closing function required for efficient chromatin assembly onto covalently closed circles of double-stranded DNA. Chromatin assembly extracts were prepared from a wild-type strain (TOP^+) and an isogenic strain (top1-top2) in which the topoisomerase I gene has been disrupted and the topoisomerase II gene bears a temperature-sensitive (*ts*) mutation that is lethal in cells grown at 37°C. Topoisomerase activity, as measured by plasmid relaxation in the presence and absence of Mg^{2+}/ATP, is readily detectable in extracts from TOP^+ but not top1-top2 cells (22). We also note that under assembly conditions, the *top2*^{ts} allele is inactive for relaxation in vitro whether or not the cells had been shifted to the restrictive temperature. The *ts* allele of topoisomerase II therefore behaves like many other mutant proteins that confer a *ts* growth phenotype and are inactive when assayed in extracts from cells grown at the permissive temperature (16, 17, 22).

Chromatin assembly was performed in these extracts by using a relaxed, closed circular plasmid template (24). Assembly was monitored by supercoiling (12) and micrococcal nuclease digestion analysis. Plasmid supercoiling is readily detected in TOP^+ extract but is severely impaired in top1-top2 extract (Fig. 1A) over a wide range of protein concentrations (Fig. 1B). Samples of template from TOP^+ and top1-top2 reactions were analyzed by electrophoresis in the presence of ethidium bromide in order to resolve nicked from covalently closed circles of DNA. Figure 1C shows that the proportion of open circular products is the same in TOP^+ and top1-top2 reactions, and so the failure to observe supercoiling in top1-top2 extract

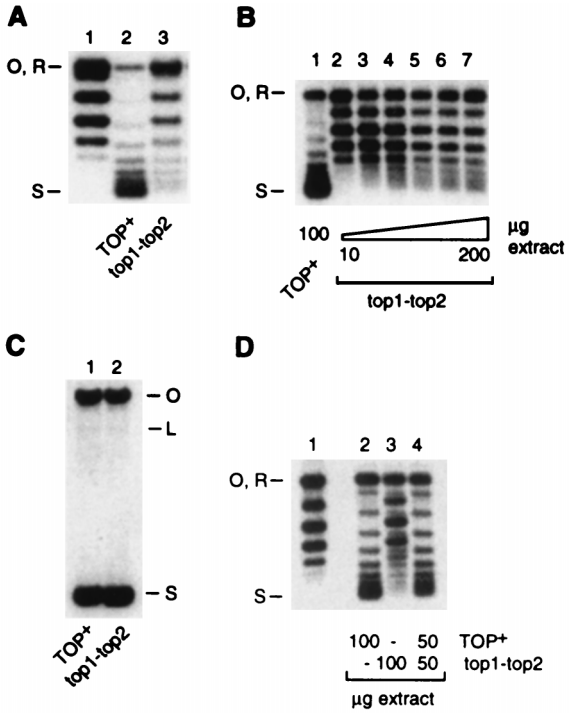
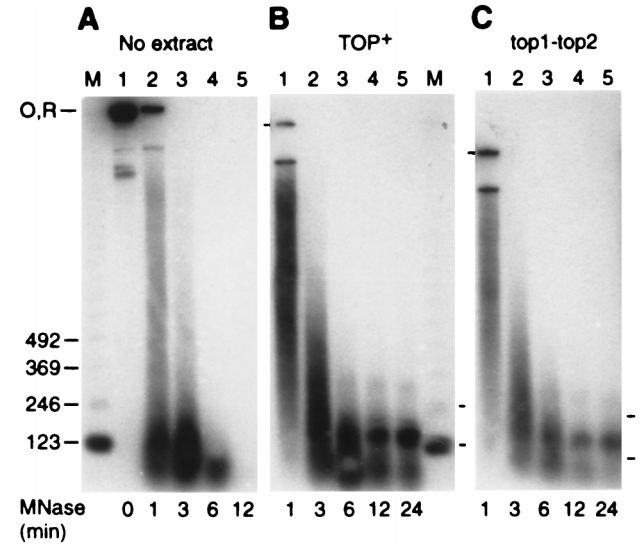


FIG. 1. A topoisomerase is required for DNA supercoiling driven by chromatin assembly in yeast whole-cell extract. Open circular (O), relaxed (R), linear (L), and highly supercoiled (S) products are resolved by native agarose gel electrophoresis. (A) Comparison of assembly-driven supercoiling in TOP^+ and top1-top2 extracts. (B) Protein titration of the top1-top2 extract. (C) Detection of open circular (nicked) DNA generated during chromatin assembly. The products of the assembly reactions were separated by agarose gel electrophoresis in the presence of 0.25 μg of ethidium bromide per ml. (D) Mixing experiment to demonstrate that top1-top2 extract does not contain an excess of an inhibitor of chromatin assembly. Assembly reactions were performed with TOP^+ and top1-top2 extracts individually and with a mixture of equal amounts of protein from both extracts.

cannot be attributed to nicking of the template. We performed a mixing experiment to test if the failure to observe supercoiling in the top1-top2 extract was due to the presence of an inhibitor of assembly in top1-top2 cells. Assembly was compared in TOP^+ extract alone, top1-top2 extract alone, and a mixture of TOP^+ and top1-top2 extracts. Figure 1D shows that the assembly capacity of the mixture of TOP^+ and top1-top2 extracts is approximately the same as that of the TOP^+ extract by itself. This result indicates that the top1-top2 extract does not contain an excess of inhibitor that would account for its low activity. Since the top1-top2 extract contains all cellular proteins except active topoisomerases I and II, we conclude that only these enzymes can efficiently provide the nicking-closing activity required for assembly-driven supercoiling in the yeast system.

The formation of nucleosomes in TOP^+ and top1-top2 extracts was further characterized by micrococcal nuclease digestion analysis (Fig. 2). As shown in Fig. 2B, template assembled in TOP^+ extract protected bands that run at approximately 165 and 330 bp in comparison to the molecular weight markers. This is the migration pattern expected for mono- and dinucleosomes, and the repeat length of approximately 165 bp corresponds to the repeat length observed for bulk chromatin in vivo in yeast (27). A monosome-sized product is also readily detected in digestions of template assembled in top1-top2 extract, and a faint dinucleosome is evident (Fig. 2C). We con-



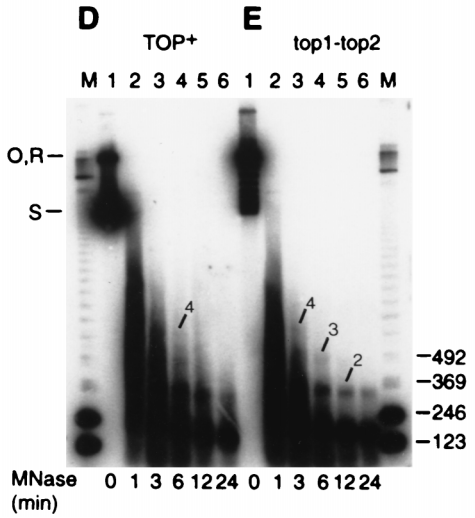


FIG. 2. Micrococcal nuclease assay for the formation of nucleosomes in wild-type and topoisomerase-deficient extracts. Assembly reactions were performed in the absence (A to C) or presence (D and E) of poly-L-glutamate and treated with micrococcal nuclease (MNase) for the indicated times. Products were detected by Southern blotting. No bands corresponding to nucleosomes are observed in the absence of yeast extract (A), whereas mononucleosomes and faint disomes are detected in the TOP$^+$ (B) and top1-top2 (C and E) extracts. Higher-order nucleosomal arrays are observed in TOP$^+$ (D) and top1-top2 (E) extracts when poly-L-glutamate is included in the assembly reaction to stabilize nucleosome ladders (see Materials and Methods). A long exposure of the gel is shown in order to demonstrate tetrasomes (labeled 4), trisomes (labeled 3), and disomes (labeled 2). Note that the difference in plasmid supercoiling between wild-type and top1-top2 extract (Fig. 1) is preserved in reactions performed with poly-L-glutamate added (lanes 1 in panels D and E). Open circular (O), relaxed (R), and highly supercoiled (S) species are labeled. The 123- and 246-bp markers (lanes M) migrate in advance of the mono- and dinucleosome, respectively.

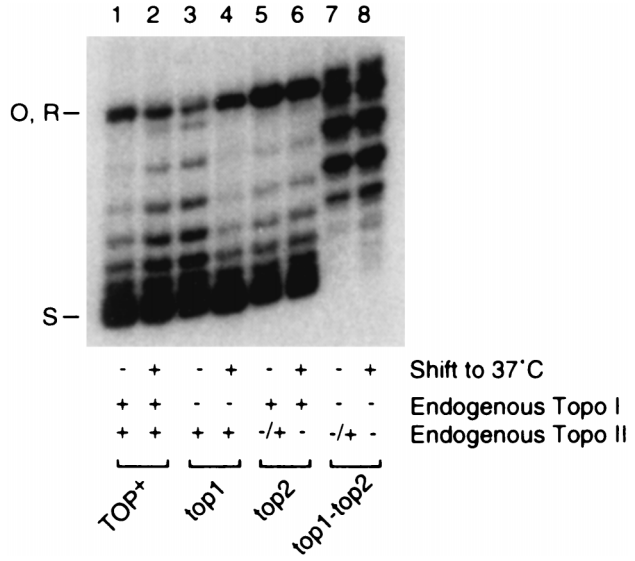


FIG. 3. Topoisomerase (Topo) I or topoisomerase II is sufficient for DNA supercoiling driven by chromatin assembly. Yeast strains were grown at room temperature and harvested before or after being shifted to 37°C for 1 h. Open circular (O), relaxed (R), and highly supercoiled (S) products are resolved by native agarose gel electrophoresis.

clude that in a biological assembly system, a normal yeast nucleosome can form on a closed circular template in the absence of topoisomerase activity. We have evidence (26) that the yeast extract contains an ATP-dependent activity, perhaps equivalent to the chromatin remodeling activity in *Drosophila* assembly extracts (28), that can disrupt nucleosomes. Nucleosomes may be more easily destabilized by this activity when they are assembled in top1-top2 extract on torsionally strained DNA. We therefore examined the nuclease digestion pattern of chromatin formed in TOP$^+$ (Fig. 2D) and top1-top2 (Fig. 2E) extracts in the presence of poly-L-glutamate, which stabilizes nucleosome arrays assembled in HeLa extracts (3). Under these conditions, a nucleosomal ladder with up to four bands is detected in both TOP$^+$ and top1-top2 extracts. This result is consistent with previous studies in *Xenopus* showing that nucleosomes can form independently of the topological state of the DNA (21).

These experiments establish a system in which it is possible to add back selected topoisomerases in order to assess the roles of these enzymes in promoting nucleosome assembly on topologically constrained templates.

Demonstration of topoisomerase I and II function in chromatin assembly by in vivo manipulations of topoisomerase activity. Using yeast strains with mutations in either *TOP1* or *TOP2* alone, we were able to determine the level of assembly-driven supercoiling in the presence of an individual topoisomerase. The experiment in Fig. 3 exploited two strains isogenic to strains TOP$^+$ and top1-top2. Strain top2 has the wild-type *TOP1* gene and the *ts* allele of *TOP2*; at the restrictive temperature, only topoisomerase I is active in this strain. Strain top1 has the *TOP1* gene disrupted, but *TOP2* is wild type; this strain contains only active topoisomerase II. From comparison of wild-type (Fig. 3, lanes 1 and 2) with top1-top2 (lanes 6 and 7) extracts, it is clear that supercoiling is severely impaired in top1-top2 extracts whether or not the cells were cultured at the restrictive temperature prior to harvesting. In top2 extract, which contains only topoisomerase I, there is a wild-type level of supercoiling (lanes 5 and 6). This is the expected result, since topoisomerase I is already known to play an important role in assembly in higher eukaryotic systems. In top1 extract, which contains only topoisomerase II, we also observe substantial supercoiling (lanes 3 and 4), in fact to about the same level as TOP$^+$ (lanes 1 and 2) and top2 (lanes 5 and 6) extracts. This result suggests that in yeast, topoisomerase II can function as efficiently as topoisomerase I to relax DNA during the assembly of nucleosomes.

This was an unexpected result, considering that careful experiments in *Xenopus* failed to detect a significant role for

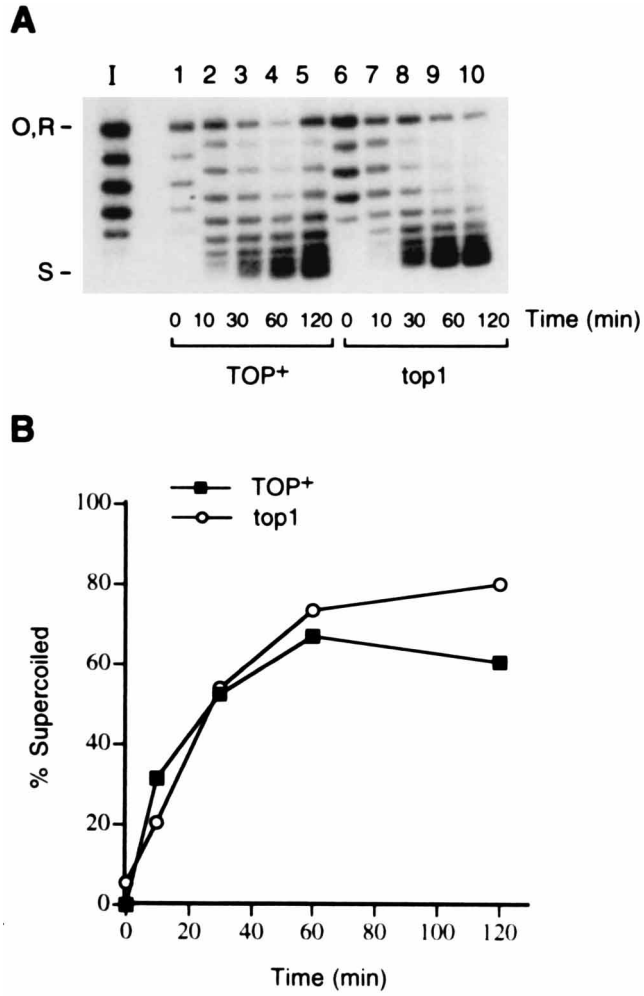


FIG. 4. Time courses of assembly-driven supercoiling in extracts from TOP$^+$ and top1 cells that were shifted to 37°C for 1 h prior to harvesting. (A) Autoradiograph showing time course of supercoiling; (B) quantitation of (A) by phosphorimager analysis. Open circular (O), relaxed (R), and highly supercoiled (S) products are resolved by native agarose gel electrophoresis. The percent supercoiled DNA was calculated as the ratio of highly supercoiled species (topoisomers migrating faster than DNA in the input [lane I]) to the total DNA per lane.

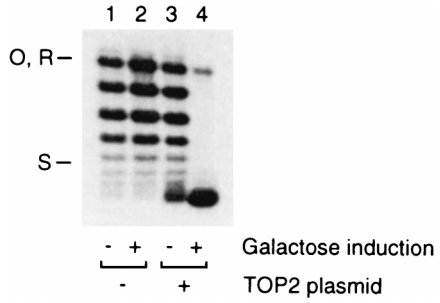


FIG. 5. DNA supercoiling is restored when wild-type topoisomerase II, under control of the *GAL1* promoter, is expressed in strain top1-top2 (lanes 3 and 4). Galactose induction of strain top1-top2 lacking the topoisomerase II overexpression plasmid does not cause any change in assembly-driven supercoiling (lanes 1 and 2). Open circular (O), relaxed (R), and highly supercoiled (S) products are resolved by native agarose gel electrophoresis.

topoisomerase II during assembly-driven supercoiling (reviewed in reference 2). We therefore analyzed the function of topoisomerase II in more detail by testing if the kinetics of supercoiling is affected when assembly is performed with topoisomerase II alone, rather than both topoisomerases I and II. Aliquots from parallel TOP$^+$ and top1 assembly reactions were stopped at various times, and the products were resolved by agarose gel electrophoresis (Fig. 4A). The level of supercoiling in this experiment was quantitated by phosphorimager analysis. The amount of supercoiled DNA in the highly supercoiled form was expressed as a percentage of the total DNA present in a lane. For quantitation, we arbitrarily designated as highly supercoiled any topoisomers that migrated faster than the top five bands in the input DNA. Visual inspection of Fig. 4A suggests that the time courses of assembly are similar in TOP$^+$ and top1 extracts. This impression is borne out by the quantitation in Fig. 4B: highly supercoiled DNA accumulates at the same rate in TOP$^+$ and top1 extracts, and the final level of supercoiling achieved in the presence of topoisomerase II alone is the same as in the presence of both topoisomerases I and II. (The higher level of assembly in top1 extract was not observed in all experiments.) From this result, we conclude that in yeast, the kinetics of assembly-driven supercoiling in the presence of topoisomerase II alone is the same as in the presence of both topoisomerases I and II.

We then tested if an in vivo add-back of wild-type topoisomerase II could rescue the assembly defect in extracts from strain top1-top2. Strain top1-top2 was transformed with a plasmid expressing the *TOP2* gene under the control of the *GAL1* promoter (32). Galactose induction of this strain, designated top1-top2/pTOP2, is expected to promote assembly-driven supercoiling if topoisomerase II can function efficiently in the context of chromatin assembly. Figure 5 shows that, as expected, assembly is poor in top1-top2 extract whether or not cells had been cultured under inducing conditions (compare lanes 1 and 2). By comparison, assembly is slightly more efficient in extracts from top1-top2/pTOP2 cells cultured under repressing conditions (lane 3), presumably due to leaky expression from the *GAL1* promoter. Full induction of the *GAL1* promoter in top1-top2/pTOP2 cells results in complete restoration of supercoiling (lane 4). This graded stimulation of supercoiling in response to graded expression of wild-type topoisomerase II in top1-top2 cells further supports our conclusion that topoisomerase II can function efficiently in the context of replication-independent chromatin assembly.

Demonstration of topoisomerase I and II function in chromatin assembly by in vitro manipulations of topoisomerase activity. We next tested if assembly-driven supercoiling could be reconstituted in vitro by adding back purified topoisomerase to top1-top2 extract. Purified topoisomerase I (calf thymus) or topoisomerase II (human) was added to top1-top2 extract, and chromatin assembly was performed under standard conditions. The reaction products were isolated, and supercoiling was analyzed by gel electrophoresis. Figure 6A shows that, as expected, topoisomerase I fully rescues the capacity of the top1-top2 extract to supercoil the template. Similarly, topoisomerase II promotes efficient supercoiling driven by chromatin assembly. This in vitro result reinforces the conclusion drawn from the experiments in Fig. 3 to 5 that both topoisomerases I and II can support chromatin assembly in the yeast system.

Although our experiments showed that the nicking-closing activity required for efficient chromatin assembly is predominantly due to the topoisomerases, we did attempt to establish conditions under which separate nicking and closing activities would substitute for the topoisomerases. Using a strain that is deficient for DNA ligase, we previously demonstrated that

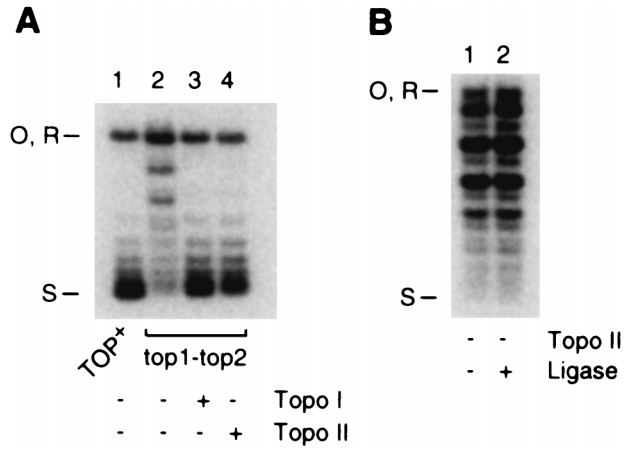


FIG. 6. DNA supercoiling driven by chromatin assembly is restored when purified topoisomerase (Topo) I (lane 3) or topoisomerase II (lane 4) is added to top1-top2 extract (A) but not when *E. coli* T4 ligase is added to top1-top2 extract (B). Open circular (O), relaxed (R), and highly supercoiled (S) products are resolved by native agarose gel electrophoresis.

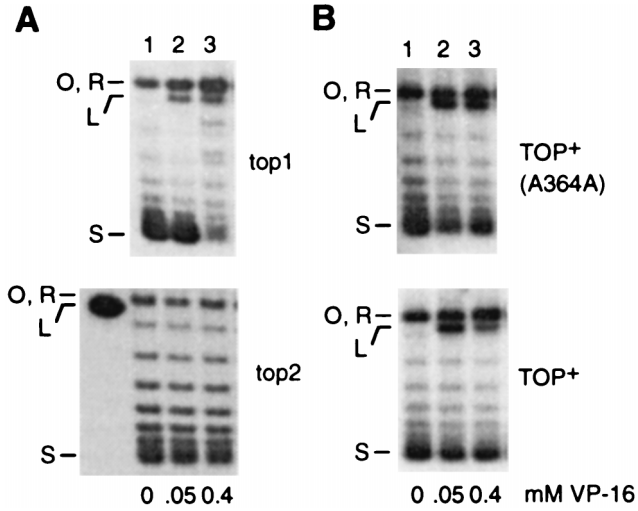


FIG. 7. Topoisomerase II has full access to DNA during chromatin assembly in the presence of topoisomerase I in wild-type extracts. Open circular (O), relaxed (R), linear (L), and highly supercoiled (S) products are indicated. Assembly was performed in the presence of the topoisomerase II inhibitor VP-16 (etoposide) as indicated. (A) Assembly reactions with top1 and top2 extracts; (B) assembly reactions with two wild-type (*TOP1 TOP2*) extracts.

there is extensive nicking of DNA during chromatin assembly in the yeast system (24). We reasoned that excess DNA ligase added to an assembly reaction, in combination with the endogenous nicking activities, might functionally substitute for the topoisomerases. T4 DNA ligase I from *E. coli* was therefore added to a top1-top2 reaction, and supercoiling was monitored by agarose gel electrophoresis. Although end-to-end ligation of linear molecules is efficient in assembly extracts even without added ligase (not shown), there was no formation of highly supercoiled products (Fig. 6B). This experiment supports the notion that a separate nuclease and ligase cannot substitute for a topoisomerase during the chromatin assembly reaction. (The same conclusion was reached in a careful analysis of assembly in *Xenopus* egg extracts [1].)

Assembly-driven supercoiling in the yeast system requires ATP (24). Since topoisomerase II is an ATP-dependent enzyme (29), we tested if ATP is required for assembly because it is a cofactor for topoisomerase II. We reasoned that assembly in a top2 extract (only topoisomerase I present) would not be inhibited by depletion of ATP if the sole function of ATP is to act as a cofactor for topoisomerase II. Supercoiling in top2 extract, however, is abolished when ATP is depleted (not shown). Apparently, then, the requirement for ATP during replication-independent chromatin assembly in yeast is not due to an obligate role for topoisomerase II in assembly. Studies of assembly in *Xenopus* also lead to the conclusion that ATP is not required because it is a cofactor for topoisomerase II (reviewed in reference 2).

Assessment of topoisomerase II activity during chromatin assembly in the presence of topoisomerase I. In *Xenopus* oocytes and eggs, chromatin assembly occurs in the presence of both topoisomerases I and II, but the contribution of topoisomerase II is limited (1, 9). The failure to detect a significant role for topoisomerase II (1) does not reflect an abnormally low content of this enzyme in these cells; oocytes actually contain a massive nuclear stockpile of active topoisomerase II (reviewed in reference 2). The results of assays using the frog system suggest that, depending on the experimental conditions, topoisomerase II may be prevented from acting during chromatin assembly when topoisomerase I is present. Although our results establish that in yeast, topoisomerase II can function during chromatin assembly when there is no topoisomerase I in a reaction (Fig. 1 to 6), we wished to determine if topoisomerase II can function during assembly in yeast in a topoisomerase I background. We used the antitumor drug VP-16 (etoposide) to measure the accessibility of topoisomerase II to DNA in a TOP^+ assembly reaction. VP-16 traps topoisomerase II on DNA after the enzyme has formed a nick or a double-strand break, generating a so-called cleavable complex in which the enzyme forms a covalent bridge between the cleaved ends of the DNA. The break in the DNA is resolved only when protein is removed. Deproteinization of a circular molecule with topoisomerase II trapped on it after breakage of both strands generates a linear product that is the length of the original circle. Removal of protein from a closed circular molecule with topoisomerase II trapped on it after single-strand cutting generates a nicked duplex. Topoisomerase II activity in the presence of VP-16 is therefore detected as the appearance of linear or nicked (open circular) products when the DNA is deproteinized and analyzed by gel electrophoresis (7, 20).

The standard control in VP-16 experiments is to perform reactions with just the solvent used to dissolve the drug (1, 9). This control, however, does not assess possible inhibitory effects of the drug that are independent of its activity against the topoisomerase. We have been able to assess potential inhibitory effects of this nature by performing assembly reactions in top1 and top2 extract in the presence of increasing amounts of VP-16 (Fig. 7A). Given the mechanism of VP-16 action, full-length linear DNA is expected to accumulate only in the reactions containing active topoisomerase II, that is, only in the top1 reactions. Figure 7A indeed demonstrates the appearance of full-length linear DNA in top1 (top panel) but not in top2 (bottom panel) reactions. We also note that the spectrum of products generated during a top2 reaction is not affected by VP-16: there is no accumulation of full-length linear DNA and no inhibition of supercoiling at the highest concentration of drug (Fig. 7A, lower panel). This result indicates that under the conditions that we use, the effects of VP-16 on assembly are almost exclusively due to inhibition of topoisomerase II rather than to nonspecific inhibition of other components of the assembly machinery.

When VP-16 was added to extracts from two TOP^+ strains, one isogenic to the top1 and top2 strains and another with different markers (A364A), we observed a significant accumulation of linear products during assembly (Fig. 7B). The proportion of full-length linear DNA generated in the presence of topoisomerase I (Fig. 7B, lanes 2 and 3) is about the same as in the absence of topoisomerase I (Fig. 7A, upper panel, lanes 2 and 3). We conclude that during assembly, topoisomerase II has full access to the DNA even in the presence of wild-type amounts of topoisomerase I.

Our results establish that the nicking-closing activity required for efficient chromatin assembly in yeast is provided by a topoisomerase and that topoisomerases I and II are equally competent to function in this capacity.

DISCUSSION

Topoisomerases I and II are the major DNA-relaxing enzymes in a eukaryotic nucleus. Previous work has established that these enzymes function interchangeably to provide relaxing activity during replication and transcription (reviewed in references 8 and 28 to 31). Here we establish that in yeast, topoisomerases I and II also function interchangeably to provide the relaxing activity required for chromatin assembly onto topologically constrained molecules of DNA.

We prepared chromatin assembly extracts from previously described yeast strains harboring mutations in one or both of the major DNA topoisomerases, mutations that either eliminate the enzyme altogether (topoisomerase I) or render it inactive under defined conditions (topoisomerase II). Chromatin assembly onto plasmid DNA added to these extracts was assessed by measuring supercoiling (agarose gel electrophoresis of DNA after protein removal) and by determining the ability of nucleosomes to protect assembled DNA from micrococcal nuclease digestion. From our initial analysis, it is clear that assembly-driven supercoiling is deficient in the top1-top2 extract (Fig. 1). The nucleosomes that do form in the absence of topoisomerase activity are normal in gross structure, as judged by micrococcal nuclease digestion analysis (Fig. 2). Therefore, as in other eukaryotes, the topoisomerases in yeast are not chromatin assembly factors (reviewed in reference 2).

Because assembly-driven supercoiling is severely inhibited in top1-top2 extract, yeast most likely does not contain a nicking-closing activity that can significantly substitute for the major topoisomerases as an accessory factor for chromatin assembly in vitro (Fig. 1). Supporting this conclusion is our observation that added DNA ligase, in combination with endogenous nicking activities in the extract, cannot promote assembly-driven supercoiling (Fig. 6B). We therefore propose that in yeast, the job of resolving torsional strain during nucleosome assembly falls almost exclusively to one or both of the major topoisomerases.

We performed experiments using genetically altered strains to determine which topoisomerase is able to support chromatin assembly in yeast. Assembly-driven supercoiling was assayed in strains with only one functional topoisomerase, either topoisomerase I or topoisomerase II (Fig. 3). This analysis clearly demonstrated that topoisomerase I alone, and topoisomerase II alone, can support efficient, assembly-driven supercoiling in yeast. A separate topoisomerase II reconstitution experiment was performed by expressing wild-type topoisomerase II in the top1-top2 background, using the inducible *GAL1* promoter. Again, assembly-driven supercoiling was supported by topoisomerase II alone (Fig. 5).

Biochemical add-back experiments also demonstrate that topoisomerase II functions efficiently during chromatin assembly: assembly-driven supercoiling was restored by adding either topoisomerase I or topoisomerase II to top1-top2 extract (Fig. 6A). From the analysis of topoisomerase mutants and the biochemical reconstitution experiments, we conclude that topoisomerase II functions as efficiently as topoisomerase I to provide the relaxing activity required for supercoiling of topologically constrained DNA molecules during chromatin assembly.

Using the specific topoisomerase II inhibitor VP-16, we were able to clearly show that topoisomerase II is catalytically active during assembly in the presence of wild-type levels of topoisomerase I (Fig. 7). From these experiments, we can estimate the activity of topoisomerase II in the presence and absence of topoisomerase I. The maximum level of topoisomerase II activity that can be obtained during assembly will be recorded in top1 extract which does not contain topoisomerase I; in this case, we will observe the maximal accumulation of full-length linear DNA due to the activity of VP-16. Since the levels of accumulation of full-length linear DNA are similar in both top1 and TOP^+ extracts treated with VP-16, we conclude that topoisomerase II is fully active during assembly in the presence of topoisomerase I.

The effect of VP-16 on the structure of DNA in a top1 reaction is dependent on the dose of VP-16 (Fig. 7A, top panel). At the low concentration of VP-16 (0.05 mM; lane 2), a significant proportion of the input DNA is cleaved on both strands at a single site, resulting in the appearance of full-length linear product. Supercoiling persists in this reaction, indicating that not all of the topoisomerase has been trapped by the drug. In contrast, at the high concentration of VP-16, there is very little supercoiling (lane 5) and the amount of template running at the position of relaxed and open circular (nicked) DNA increases. This result indicates that at 0.4 mM VP-16, all of the topoisomerase II is sequestered on DNA in cleavable complexes and that in some cleavable complexes (those running at the position of open and relaxed circles), only one strand of the duplex is cut.

Based on the results presented here, we suggest that topoisomerase II can efficiently provide the relaxing activity for chromatin assembly in vivo. We have shown that topoisomerase II alone can provide the relaxing activity required for efficient assembly in vitro whereas separate nicking and closing activities cannot. Therefore, it is likely that in vivo, topoisomerase II provides the relaxation activity required for chromatin assembly in a topoisomerase I null mutant. Our in vitro experiments further show that the capacity of topoisomerase II to participate in chromatin assembly is not inhibited by the presence of topoisomerase I (Fig. 7). Based on this observation, we propose that in a TOP^+ cell, both enzymes significantly participate in assembly. The actual contribution of each enzyme to assembly in a TOP^+ cell may largely depend on the relative abundance of these enzymes at any particular site in the genome. For example, a high concentration of topoisomerase I is found in the nucleolus, where rDNA is transcribed (10, 14, 19). Chromatin assembly at the rDNA locus may therefore be promoted by topoisomerase I rather than topoisomerase II. This possibility is supported by the observation that the stability of nucleosomes in transcribed rDNA is highly sensitive to depletion of topoisomerase I but not topoisomerase II (6). Since topoisomerase II is rather evenly distributed throughout the interphase nucleus in yeast (18), this enzyme may significantly participate in assembly at all locations except the nucleolus. Extending these speculations to the situation in oocytes, eggs, and embryos (1, 4), the reported dominance of topoisomerase I over topoisomerase II may reflect the relative abundance of topoisomerases I and II in these cell types rather than specific

recruitment of topoisomerase I by the chromatin assembly machinery.

In summary, our results show that in terms of the ability to provide DNA relaxation activity during chromatin assembly, DNA topoisomerase II appears to be completely redundant with topoisomerase I. Therefore, both topoisomerases I and II function efficiently during three principal activities in the nucleus that generate torsional strain in DNA: chromatin assembly, transcription, and replication.

ACKNOWLEDGMENTS

We thank our coworkers for lively discussions and Darren Hockman for advice on Southern blotting. Rolf Sternglanz is thanked for yeast strains. James Wang kindly provided plasmid YEpTOP2-PGAL1, and James Berger made useful suggestions concerning the galactose induction of this plasmid. Etoposide was a gift from A. M. Casazza and S. J. Luciana, Bristol-Myers Squibb, Princeton, N.J.

This work was supported by the Alberta Heritage Foundation for Medical Research. M.C.S. is a Scholar of the Medical Research Council, and W.I.G. has been supported by a University of Alberta Ph.D. scholarship and a studentship from the AHFMR.

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