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DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: Topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication

(yeast/cell cycle/catenated dimers)

STEPHEN DINARDO*, KAREN VOELKEL, AND ROLF STERNGLANZ†

Department of Biochemistry, State University of New York, Stony Brook, NY 11794

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ABSTRACT **A temperature-sensitive DNA topoisomerase II mutant of the yeast *Saccharomyces cerevisiae* has been identified. Genetic analysis shows that a single recessive nuclear mutation is responsible for both temperature-sensitive growth and enzymatic activity. Thus, topoisomerase II is essential for viability and the mutation is most probably in the structural gene. Experiments with synchronized mutant cells show that at the nonpermissive temperature cells can undergo one, and only one, round of DNA replication. These cells are arrested at medial nuclear division. Analysis of 2-μm plasmid DNA from these cells shows it to be in the form of multiply intertwined catenated dimers. The results suggest that DNA topoisomerase II is necessary for the segregation of chromosomes at the termination of DNA replication.**

DNA topoisomerases are enzymes that catalyze the concerted breakage and rejoining of DNA backbone bonds (1). Topoisomerases can be divided into several categories, depending on their source and mode of action (reviewed in ref. 2). Eukaryotic type 2 topoisomerases, the subject of this paper, can catalyze several different DNA isomerization reactions, including the relaxation, catenation, decatenation, knotting, and unknotting of closed double-stranded DNA circles (3–5). Although the eukaryotic type 2 DNA topoisomerases are fairly well characterized *in vitro*, nothing is known about their *in vivo* roles. It has been suggested that these enzymes might be involved in initiation of DNA replication (3) or in the segregation of daughter DNA molecules at the termination of DNA replication (6, 7).

The properties of the yeast topoisomerases are quite similar to their counterparts from mammalian cells (8, 9). To learn more about the role of eukaryotic DNA topoisomerases, we began a search for yeast topoisomerase mutants. We reasoned that a study of the phenotypes of such mutants would lead to a greater understanding of the *in vivo* roles of these enzymes in all eukaryotic cells. Here we describe the identification and characterization of a temperature-sensitive (ts) yeast DNA topoisomerase II mutant. We show that it is defective in the segregation of daughter chromosomes at the termination of DNA replication.

MATERIALS AND METHODS

Yeast Strains. The collection of ts mutants of *Saccharomyces cerevisiae* (10) was generously provided by C. McLaughlin. The parent strain of the collection is A364A:*MATa ade1 ade2 ura1 lys2 tyr1 his7 gal1*. Strain 14-16 from the collection was found to have a *top2*(ts) mutation. It was backcrossed three times to derivatives of S288C, either 946:*MATa ura3-52 suc2* or 947:*MATα ade2-101 ura3-52* provided by D. Shortle. Strain SD1-4 *MATa ade1 ade2 ura3-52, top2-1*(ts), a segregant from the last backcross, was used for phenotype studies.

Yeast Growth Conditions. Medium for growth of yeast was YPD (11) or YM-5 (12). The *in vivo* uniform labeling experiments were carried out essentially as described (12), except that [5,6-^{3}H]uracil at 3 μCi/ml (41 Ci/mmol; 1 Ci = 37 GBq) was used. Cells were synchronized with α-factor pheromone as described (13). Nuclear staining was carried out as described (14), except that Hoechst 33258 dye was used instead of DAPI. Progress through the yeast cell cycle was monitored morphologically by phase-contrast microscopy (13).

Topoisomerase Assays. Cells were grown in 25 ml of YPD medium at 25°C. During exponential growth cultures were shifted to 37°C for 20 min, chilled, centrifuged, washed with cold H_2O, and recentrifuged. The cell pellet was resuspended in 0.5 ml of yeast lysis buffer (20 mM Tris·HCl, pH 7.5/1 mM Na_2EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/500 mM KCl/10% glycerol). One-third volume of glass beads (Sigma, type IV, 250–300 μm) was added and the cells were lysed by brief sonication. The lysate was centrifuged for 10 min in a desk top centrifuge. One microliter of the supernatant (undiluted or diluted in yeast lysis buffer plus 100 μg of bovine serum albumin per ml) was used for topoisomerase assays. DNA topoisomerase I assays were in 10 μl total volume containing 20 μg of supercoiled pBR322 DNA per ml, 20 mM Tris·HCl (pH 7.5), 10 mM Na_2EDTA, 1 mM dithiothreitol, 30 μg of bovine serum albumin per ml, and 150 mM KCl. DNA topoisomerase II assays (5) were in 10 μl total volume containing 20 μg of kinetoplast DNA per ml, 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM Na_2EDTA, 1 mM dithiothreitol, 30 μg of bovine serum albumin per ml, 150 mM KCl, and 0.4 mM ATP. Incubation for both topoisomerase I and II assays was at 37°C for 30 min. Samples were electrophoresed in 0.7% agarose gels (15). In experiments in which DNA was treated with partially purified yeast DNA topoisomerase I or topoisomerase II the incubation was at 30°C for 30 min. Nicking with DNase I was in the presence of ethidium bromide for 2–7 min at 30°C with 20 μg of added sonicated calf thymus DNA per ml (16).

Preparation of Nuclear Extracts and Fractionation by Hydroxylapatite. Crude nuclei were prepared from spheroplasts as described (17) and resuspended in buffer A (17) with 4 mM Na_2EDTA and 1 mM phenylmethylsulfonyl fluoride. The suspension was stirred gently for 15 min at 4°C, and then nuclei were lysed by addition of an equal volume of 2 M NaCl/50 mM Tris·HCl, pH 7.5/ 1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride. Debris and high molecular

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Abbreviations: ts, temperature-sensitive; SV40, simian virus 40.

*Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143.
†To whom reprint requests should be addressed.

weight DNA was pelleted by centrifugation for 1 hr at 100,000 × *g*. The supernatant was adjusted to 10% glycerol. Residual DNA was precipitated by polyethylene glycol, and fractionation of the nuclear extract by hydroxylapatite column chromatography was carried out as described (18).

DNA Isolation and Southern Blot Analysis. Total yeast DNA was prepared as described (11). For minichromosome isolation, cultures were inoculated with G_0 cells (19) into 1 liter of YPD medium prewarmed to 37°C. Cells were harvested after 5 hr and minichromosomes were prepared as described (20). Centrifugation was for 7 hr at 17,000 rpm in a Beckman SW 27 rotor. Fractions (1.5 ml) were collected by puncturing the bottom of the tube with an 18-gauge needle. Monomer and head-to-tail dimer minichromosomes are separated in the sucrose gradient, and multiply intertwined catenated dimers sediment at about the position of head-to-tail dimer. High-resolution 0.8% agarose gel electrophoresis was as described (6, 7), except that $NaDodSO_4$ was omitted and electrophoresis was for 44 hr. Southern transfers and hybridization were carried out as described (21, 22).

RESULTS

Identification of a DNA Topoisomerase II Mutant. A collection of independently isolated ts mutants of the yeast *S. cerevisiae* (10) was screened for DNA topoisomerase defects. Details of the procedure are in *Materials and Methods*. After screening about 150 mutants, a strain deficient in DNA topoisomerase II was identified. Fig. 1 compares DNA topoisomerase activity for the wild-type, strain A364A, and for the mutant, strain 14-16. It can be seen that topoisomerase I activity is about the same for both strains, whereas topoisomerase II activity is much reduced in strain 14-16.

The mutant strain was mated with a wild-type strain of opposite mating type and the resulting diploids sporulated and were subjected to standard tetrad analysis. The ts phenotype and the topoisomerase II defect cosegregated 2:2, indicating that a single nuclear mutation is responsible for both phenotypes. This conclusion held true for a total of nine tetrads from four successive backcrosses of the mutant with wild type. Also, temperature-resistant revertants of the mutant regain enzymatic activity.

When the mutant is grown at the permissive temperature without a shift to 37°C and then assayed at 25°C and 37°C, it exhibits almost normal activity at 25°C and at most 1/10th as

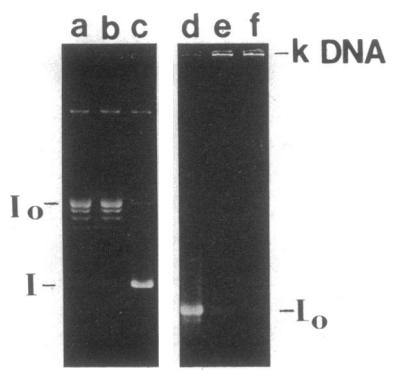

FIG. 1. DNA topoisomerase I and topoisomerase II assays of wild-type (A364A) and *top2*(ts) mutant (strain 14-16). Lanes a–c, topoisomerase I assays; lanes d–f, topoisomerase II assays. Lanes a and d, wild-type extract; lanes b and e, *top2*(ts) extract; lanes c and f, control (no extract added). Topoisomerase I activity in wild-type and mutant extracts produced fully relaxed DNA (I_0, lanes a and b) from substrate supercoiled pBR322 DNA (I, lane c). The catenated network of kinetoplast DNA (kDNA) remains at the origin (lane f), but the monomer circles (I_0) liberated by topoisomerase II activity enter the gel. The wild-type extract exhibits topoisomerase II activity (lane d), whereas the *top2*(ts) extract does not (lane e).

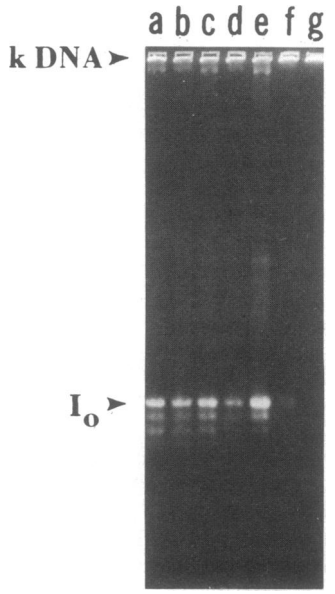

FIG. 2. Temperature-sensitivity profile of DNA topoisomerase II activity from *top2*(ts) mutant after hydroxylapatite chromatography. Lanes a, c, and e, wild type; lanes b, d, and f, mutant; lane g, control (no enzyme added). Lanes a and b, 24°C; lanes c and d, 30°C; lanes e and f, 37°C. Other symbols as in Fig. 1.

much activity at 37°C. This ts enzymatic activity is also seen when the mutant enzyme is purified about 20-fold on a hydroxylapatite column (Fig. 2). The combination of these genetic and biochemical data strongly suggests that the mutation originally present in strain 14-16 is in the structural gene for topoisomerase II and that the enzyme is essential for viability. In work to be described elsewhere, we have isolated a DNA plasmid from a yeast gene library that complements the *top2*(ts) mutation. Using this clone, we have genetically mapped the *TOP2* gene to chromosome XIV, near the *MET4* gene.

Phenotypes of the DNA Topoisomerase II Mutant. Strain SD1-4 carrying the ts topoisomerase II [*top2*(ts)] mutation was used for phenotype studies. RNA and DNA synthesis were monitored by labeling cells with [^{3}H]uracil. Experiments with asynchronous cultures suggested that the mutant had a defect in DNA replication but it was difficult to know if this was a primary phenotype. Experiments with cells synchronized with the yeast pheromone, α-factor were more informative. This agent blocks cells of *a* mating type in the G_1 phase of the cell cycle. Removal of the α-factor releases the cells from the G_1 block and allows a single, moderately synchronous cell cycle. Wild-type and mutant cells were released from the G_1 block at either 24°C or 38°C and DNA synthesis was monitored. As seen in Fig. 3, both strains behave identically at 24°C with an S phase of DNA synthesis occurring at the expected time. At the nonpermissive temperature, 38°C, wild-type cells also go through a normal S phase and then continue into a second cell cycle. In contrast, the topoisomerase II mutant at 38°C exhibits a normal initial S phase, doubling its DNA content, but then it stops DNA synthesis completely.

To determine approximately where in the cell cycle the *top2*(ts) mutant had arrested, samples of the synchronized culture were examined by phase-contrast microscopy at various times after DNA synthesis had stopped. At 135 min most of the cells exhibited a single terminal morphology. Specifically, about 80% of the cells had a single large bud, corresponding well with the observed increase in DNA synthesis (Fig. 3). Nuclear staining indicated that these cells were arrested with the nucleus in the neck between the

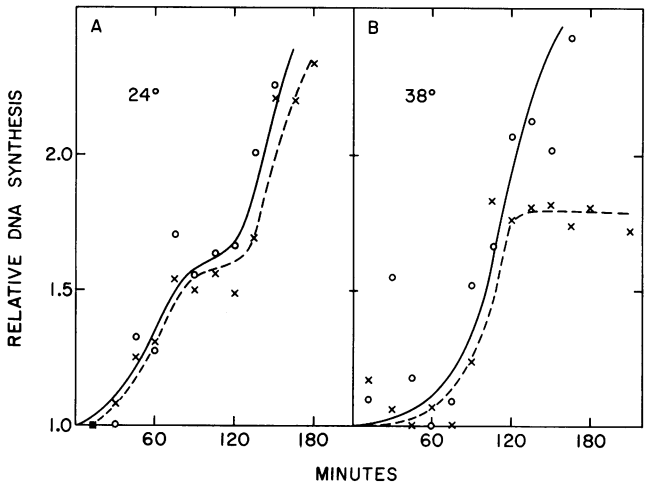

FIG. 3. DNA synthesis in synchronized cultures of wild-type and *top2*(ts) strains. Cells growing in YM-5 medium at 25°C in the presence of [^{3}H]uracil (3 μCi/ml) were arrested by the addition of yeast α-factor. The α-factor was removed and cells were resuspended in YM-5 medium with 3 μCi of [^{3}H]uracil per ml and incubated at 24°C (*A*) or 38°C (*B*). Samples were removed at various times and the amount of ^{3}H incorporated into DNA was determined (12). —○—○—, Wild type; ---×---×---, *top2*(ts).

mother cell and its daughter bud—i.e., at medial nuclear division. At later times, about 15% of these cells had rebudded without cell division, giving rise to aberrantly budded forms. These results argue that topoisomerase II is required in the yeast cell cycle at some point between the α-factor arrest point in G_1 and medial nuclear division. Exponentially growing cells shifted to the nonpermissive temperature do not show a single terminal morphology, suggesting that topoisomerase II may also be required elsewhere in the cell cycle.

A Defect in Segregation of Daughter DNA Molecules. The results with synchronized cells described above as well as the known *in vitro* decatenation activity of DNA topoisomerase II suggested to us that the *top2*(ts) mutant might have a defect in termination of DNA replication. To test this possibility we examined the structure of 2-μm DNA in the *top2*(ts) strain. The 2-μm plasmid is present in most yeast strains at about 50 copies per cell (23). Replication of the plasmid is under the same cell cycle control as chromosomal DNA. Each molecule is replicated only once, early in S phase (24), and replication requires the same *cdc* gene products as does chromosomal DNA (25).

Strain SD1-4 *top2*(ts) and a wild-type strain were synchronized with α-factor and released from G_1 arrest as in Fig. 3. After completion of the first S phase, the structure of the 2-μm DNA was analyzed by gel electrophoresis. The results in Fig. 4 show that 2-μm DNA isolated from wild-type cells at 24°C and 38°C and from the mutant at 24°C migrates at the expected positions, mainly monomer and head-to-tail dimer supercoiled circles with a small fraction of nicked circles (Fig. 4, lanes a–c). On the other hand, 2-μm DNA from the mutant at 38°C migrates predominantly between form I and form II DNA as an unusual series of closely spaced bands (Fig. 4, lane d). Very little supercoiled and nicked monomer or head-to-tail dimer is seen. These bands are too closely

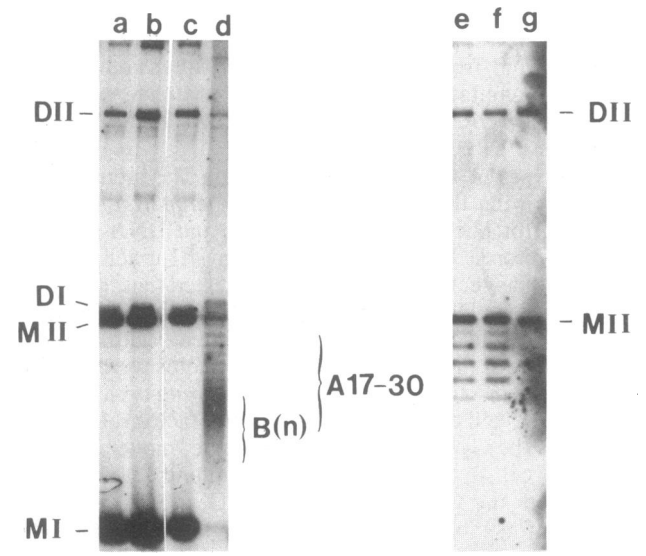

FIG. 4. Structure of 2-μm DNA from wild-type and *top2*(ts) strains. Strains were synchronized with α-factor and total DNA was prepared from samples taken at 120 min (24°C) or 165 min (38°C) after removal of α-factor. The DNA was electrophoresed and analyzed by Southern blot hybridization with ^{32}P-labeled 2-μm DNA as a probe. Lane a, wild type, 24°C; lane b, wild type, 38°C; lane c, *top2*(ts), 24°C; lane d, *top2*(ts), 38°C. Samples in lanes e–g were treated with yeast DNA topoisomerase II before electrophoresis. Lane e, wild type, 38°C; lane f, *top2*(ts), 24°C; lane g, *top2*(ts), 38°C. MI and DI, supercoiled monomer and supercoiled head-to-tail dimer, respectively. MII and DII, nicked monomer and nicked head-to-tail dimer, respectively. A17–30, catenated dimers, of catenation linking nos. 17 through 30, wherein both participating DNA rings are nicked. B(*n*), catenated dimers, of undetermined (but high) catenation linking number, wherein one DNA ring is nicked and the other is closed.

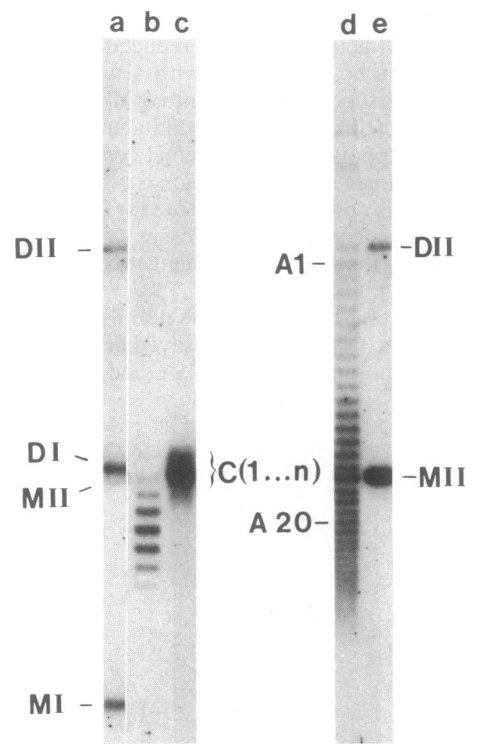

FIG. 5. Structure of 2-μm DNA from minichromosomes isolated from a *top2*(ts) mutant. Lane a, wild-type, monomer and head-to-tail dimer fractions pooled; lane b, *top2*(ts) as in lane c, but topoisomerase II treated; lane c, *top2*(ts), catenated dimer fractions; lane d, as in lane c, but nicked with DNase I; lane e, as in lane d, but topoisomerase II treated. C(1...*n*), multiply intertwined catenated dimers of catenation linking nos. 1 through *n*, wherein both DNA rings are closed. A1 and A20, catenated dimers of catenation linking nos. 1 and 20, respectively. The position A1 was verified by determining the position of a singly catenated dimer produced by the action of DNA gyrase on monomer 2-μm DNA, as described (26). Other symbols as in Fig. 4.

spaced to be monomer closed circles simply differing in linking number (compare with the bands in Fig. 4, lanes e and f), and they do not have the correct migration for monomer circles containing knots of increasing complexity.

When DNA from wild-type at 38°C or the mutant at 24°C is treated with partially purified yeast DNA topoisomerase II, the 2-μm DNA is converted to covalently closed, relaxed circles, as expected (Fig. 4, lanes e and f). When DNA from the mutant at 38°C is similarly treated with topoisomerase II, mainly nicked circles and some closed, relaxed circles are produced (Fig. 4, lane g, and data not shown).

These results suggested to us that 2-μm DNA from the mutant might be in the form of multiply intertwined catenated dimers. Sundin and Varshavsky have described such forms in their studies of simian virus 40 (SV40) DNA replication intermediates (6). Using hypertonic growth medium, they observed an accumulation of SV40 DNA catenated dimers with up to 20–25 intertwinings of one molecule about the other (7). They demonstrated that three types of catenated dimers could be distinguished by gel electrophoresis: C forms, with both DNA circles covalently closed; B forms, with one circle closed and one open; and A Forms, with both circles open. The series of closely spaced bands seen in Fig. 4, lane d, could represent A form multiply intertwined catenated dimers. Each band in the series could correspond to a dimer with a discrete number of intertwinings.

To demonstrate this, we prepared 2-μm DNA from isolated minichromosomes (20). This protocol yielded DNA relatively free of single-strand breaks, enabling us to analyze the C form catenated dimers. Fig. 5, lane a, shows DNA from wild-type cells, migrating mainly as supercoiled monomer and head-to-tail dimer, as expected. Fig. 5, lane c, shows 2-μm DNA from the mutant, in this case migrating as a broad band labeled C(1...*n*). This DNA is converted to covalently closed, relaxed monomer circles after treatment with topoisomerase II (Fig. 5, lane b). Form C multiply intertwined catenated dimers can be converted into A forms by nicking each of the two DNA duplexes. Indeed, extensive nicking of the DNA displayed in Fig. 5, lane c, with DNase I gives rise to a discrete series of bands, migrating at positions labeled A1 through A20 in Fig. 5, lane d. As expected, decatenation of these A forms with topoisomerase II releases nicked monomer circles (Fig. 5, lane e).

Nicking one of the rings of a catenated dimer should produce a B form molecule containing two different topological domains, one open and one closed. Our evidence for the production of B forms is shown in Fig. 6. A preparation of C form catenated dimers was lightly nicked and is displayed in Fig. 6, lane c. Decatenation of this material produces both nicked monomer circles and covalently closed, relaxed monomer circles (Fig. 6, lane d). The nicked circles are derived mainly from the A forms as in Fig. 5, lanes d and e. The covalently closed circles must be derived from the material labeled B(*n*), as this is the only difference seen upon comparing the DNA patterns in Fig. 5, lane d, versus Fig. 6, lane c.

Another preparation of DNA that was slightly nicked during isolation, and hence contains both C and B forms, is displayed in Fig. 6, lane f. The B(*n*) forms are clearly evident since no A forms are present to obscure this region of the gel. Decatenation of this DNA by topoisomerase II releases both covalently closed and nicked monomer circles (Fig. 6, lane g). The production of nicked circles here is in striking contrast to the absence of any nicked circular DNA produced by topoisomerase II treatment of pure C form DNA (compare Fig. 6, lane g, with Fig. 5, lane b). The nicked monomer circles must be derived from the open circle domain of the B forms.

DISCUSSION

The most striking phenotype we have detected in the *top2*(ts) mutant is a defect in the segregation of daughter chromosomes at the termination of DNA replication. We have

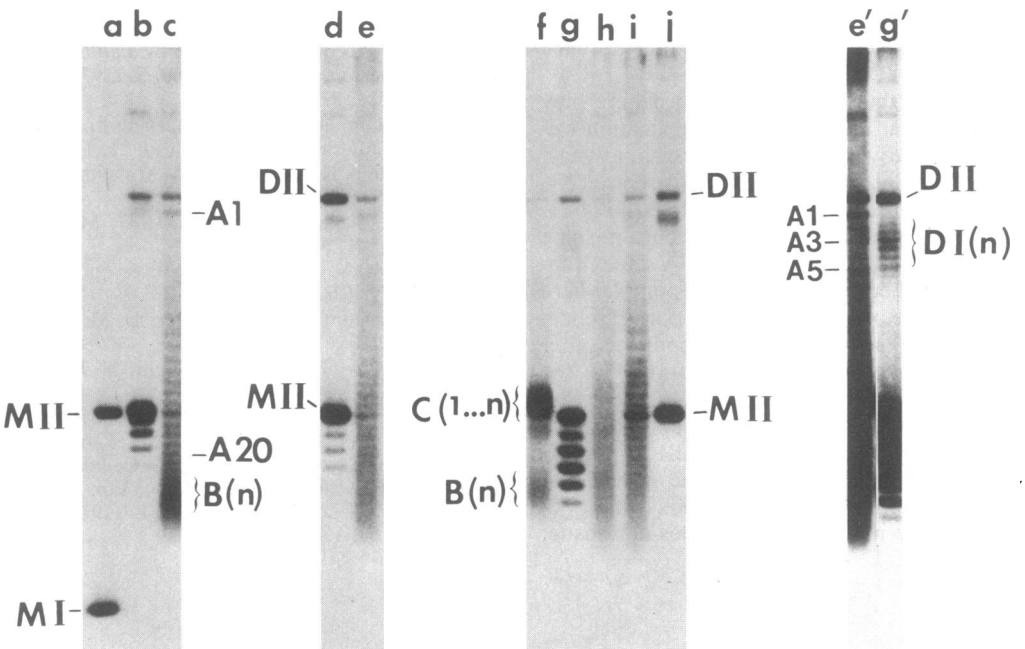

FIG. 6. Relationship between A, B, and C form catenated dimers of 2-μm circle DNA from *top2*(ts) mutant. DNA was prepared from minichromosomes as in Fig. 5. Lane a, wild-type, monomer fraction; lane b, wild-type, monomer and head-to-tail dimer fractions pooled, treated with topoisomerase I; lane c, *top2*(ts), catenated dimer fraction, nicked with DNase I; lane d, as in lane c, but treated with topoisomerase II; lane e, as in lane c, but treated with topoisomerase I; lane f, *top2*(ts), catenated dimer fraction from another minichromosome preparation; lane g, as in lane f, but treated with topoisomerase II; lane h, as in lane f, but treated with topoisomerase I; lane i, as in lane f, but nicked with DNase I; lane j, as in lane i, but treated with topoisomerase II. Lanes e′ and g′ are longer exposures of lanes e and g, respectively. A1, A3, and A5, catenated dimers of catenation linking nos. 1, 3, and 5, respectively. DI(*n*), a family of relaxed topoisomers of head-to-tail dimer, wherein each topoisomer differs by one in the number of superhelical turns. The spacing between catenated dimer topoisomers A1 through A5 (lane e′) is twice the spacing seen for the head-to-tail dimer topoisomers (lane g′), just as expected (26). Other symbols as in Figs. 4 and 5.

presented evidence that 2-μm circle DNA accumulates as multiply intertwined catenated dimers after one period of DNA synthesis (S phase). This result is not peculiar to 2-μm DNA. We have observed that a DNA plasmid containing yeast chromosomal sequences (ARS1, CEN3, and LEU2), but no 2-μm circle sequences, also accumulates as multiply intertwined catenated dimers (data not shown). Sundin and Varshavsky have previously described the appearance of multiply intertwined catenated dimers as intermediates arising at the terminal stage of SV40 DNA replication (6, 7). These authors proposed that a type 2 DNA topoisomerase might be responsible for segregating daughter chromosomes by decatenation. Evidently in both yeast and mammalian cells bidirectional replication of circular genomes results in highly intertwined catenated dimers.

A proposed mechanism has been described for the formation of multiply intertwined catenated dimers (6, 7). Briefly, DNA replication forks are established at a specific site, the origin, and proceed bidirectionally around the circular genome. As the replication forks approach each other, fork movement halts, leaving 200–300 base pairs of unreplicated parental duplex. This hesitation may result from the steric exclusion of a "swivelase" from the region between converging forks. Up to this point the postulated swivelase has been responsible for unrestrained fork movement. The catenated dimer forms as the residual Watson–Crick turns of the parental duplex are unwound and replicated. Each helical turn replicated in the absence of the postulated swivelase is converted into a topologically equivalent catenation turn or duplex intertwining. We find an average of 20–30 intertwinings in the A forms depicted in Fig. 4, lane d. Remarkably, a similar average catenation linking number was found for SV40 catenated dimers (7). Apparently the constraints imposed by the replication fork machinery are similar in yeast and mammalian cells.

For both 2-μm DNA (Figs. 5 and 6) and SV40 DNA (6, 7), the multiply intertwined catenated dimers can be isolated as C forms, with no single-strand breaks. Evidently DNA synthesis and ligation can proceed to completion in the absence of decatenation.

It is important to note that the steps outlined here may apply equally well to linear chromosomes. Replication origins are spaced about every 36 kilobase pairs along yeast chromosomal DNA as judged by electron microscopy (27). Since the average yeast chromosome is about 800 kilobase pairs (28), there are several sites along each chromosome where bidirectional DNA replication initiates. A direct consequence of this is that there will be several points where replication forks converge. At each point of convergence, 20–30 intertwinings of one sister chromatid about the other will result. Rotation of one long sister chromatid about the other may be constrained inside the cell to the extent that topoisomerase II is required to disentangle the sister chromatids. This in fact is an explanation for arrest at nuclear division of synchronized *top2*(ts) cells. In the absence of topoisomerase II disjunction of sister chromatids cannot occur properly and therefore nuclear division does not ensue. One piece of evidence consistent with this hypothesis is that chromosome loss is about 30-fold higher in a *top2*(ts) mutant than in wild-type, when grown at the semipermissive temperature, 30°C (L. Hartwell, personal communication).

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