Title: **“DNA catenation reveals the dynamics of DNA topology during replication”**

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Running Title: **Catenane's quantification**

Key words: ***Saccharomyces cerevisiae* / Replication / Supercoiling / Catenation / 2D gels /**

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ABSTRACT

Two-dimensional agarose gel electrophoresis is the method of choice to identify and quantify all the topological forms DNA molecules can adopt *in vivo*. Here we describe the materials and protocols needed to analyze catenanes, the natural outcome of DNA replication, in *Saccharomyces cerevisiae*. We describe the formation of pre-catenanes during replication and how inhibition of topoisomerase 2 leads to the accumulation of intertwined sister duplexes. This knowledge is essential to determine how replication forks blockage or pausing affects the dynamics of DNA topology during replication.

INTRODUCTION

DNA catenation is the natural outcome of replication. Due to the double-stranded nature of DNA, for the genetic code to be copied the two parental strands must separate and this melting causes an increase of torsional tension ahead of the fork (1). The accumulation of this positive (+) torsional tension is exacerbated when two forks progressing in opposite directions approach each other during termination. At some point topoisomerases simply have not enough space to bind and relax the DNA that remains to be replicated. It was suggested one way to solve this problem is swiveling of the forks (Figure 1) to diffuse the (+) torsional tension to the replicated region where it adopts the form of right-handed (RH) precatenanes (2). Relaxation of the torsional tension ahead of the forks permits DNA replication to go on. Once replication is over, topoisomerase 2 would eliminate the remaining catenanes to allow sister duplexes segregation. There is still controversy, though, as to whether fork swiveling occurs all the way during replication (3) or only at the end as termination approaches (4). In any case, the final number of intertwinings is directly related to the proficiency of replication progress. In other words, replication fork blockage or pausing affects precatenation and consequently the final number of catenane's intertwinings. Under normal conditions replication and decatenation progress very fast and the only way to visualize catenanes is to inhibit topoisomerase 2 (5-10). The inhibition of topoisomerase 2 can be accomplished exposing the cells to specific drugs or using conditional mutants. The latter method is preferable since drugs have secondary effects and usually cause single- and double-stranded DNA breakage (3). The best method to identify catenated molecules so far is two-dimensional (2D) agarose gel electrophoresis (Figure 2A). Unreplicated forms of intact circular DNA generate covalently closed monomers (CCCm) and dimers (CCCd) as well as open circles of

monomers (OCm), dimers (OCd) and knotted molecules (Knm). All these forms are clearly identified in 2D gels (Figure 2A). The inhibition of DNA segregation leads to the accumulation of three forms of catenated molecules (5-10): CatAs, formed by two intertwined OCs; CatBs, formed by two intertwined circles one of which is an OC and the other a CCC; and CatCs, formed by two intertwined CCCs. Exposure of all these isolated forms to a restriction endonuclease that causes single-stranded breakage (nicks) converts all catenated forms to CatAs where each dot of increased mobility in the arc corresponds to the addition of one catenation. The immunograms shown to the right in Figure 2 correspond to intact circular forms of pYAC_MEM isolated from a top2-td strain of *Saccharomyces cerevisiae* (7, 11). It is clearly seen that minichromosomes isolated from cells grown at the permissive temperature mainly occurs as CCCm and CCCd (Figure 2B). Nicking of these molecules converts all of them to OCm and OCd (Figure 2C). Minichromosomes isolated from cells grown at the restrictive conditions occurs as CCCm but also as catenated forms: CatAs, CatBs and CatCs (Figure 2D). Finally, nicking of these molecules converts all catenated forms to CatAs (Figure 2E) allowing the precise quantification of the intertwined forms, over 50 in the corresponding immunogram.

MATERIALS

1. Yeast transformation

- YEP medium (1% yeast extract, 2% peptone, 60 mg/mL adenine, pH 6.5).
- 40% raffinose (Raf)
- TE (100:10 Tris HCl-EDTA) pH 7.5
- 1M lithium acetate (LiAc) pH 7.5 stored at 4°C
- 10 mg/mL sonicated and denatured salmon sperm DNA stored at 4°C
- 50% PEG (polyethylene glycol 6,000) stored at 4°C
- DMSO (dimethyl sulfoxide)
- Appropriate selective medium
- Appropriate selective medium agar plates with 2% Raf

2. Transformation checking

a) DNA fast isolation

- Appropriate selective médium
- 40% Raf
- Breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0 and 1mM EDTA pH 8.0)
- P-CIA 25:24:1 (phenol- chloform isoamyl alcohol) stored at 4°C
- Glass beads (0.5 mm of diameter)
- FastPrep®-24 Instrument
- TEN₁₀₀ (100 mM NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0).
- CIA 24:1 (chloform: isoamyl alcohol) stored at 4°C
- 100% ethanol
- 10 mg/mL RNase A stored at -20°C

b) Unidimensional agarose gel electrophoresis

- Agarose (Seakem LE, Lonza)
- 5x TBE (Tris-Borate-EDTA) 0.445 M Tris base, 0.445 M boric acid and 0.01 M EDTA
- 10× gel loading buffer: 0.1 % xylene cyanol, 0.1 % bromophenol blue, 30 % glycerol and 10 mM EDTA, pH 8.0

c) Southern blotting

- 37% hydrochloric acid fuming
- 4 M sodium hydroxide
- 20× SSC (saline sodium citrate): 3 M sodium chloride and 0.3 M tri-sodium citrate
- Whatman 3MM paper
- Zeta-Probe blotting membranes (Bio-Rad)

d) Non-radioactive hybridization

- Digoxigenin-High Prime kit (Roche)

- 20× SSPE (saline sodium phosphate EDTA): 3.6 M sodium chloride, 0.2 M disodium phosphate, 20 mM EDTA
- 10 % Blotto (non-fat powdered milk)
- 20 % SDS (sodium dodecyl sulfate)
- 20 % dextran sulfate stored at 4°C
- 10 mg/mL sonicated and denatured salmon sperm DNA stored at 4°C
- 20× SSC (saline sodium citrate): 3 M sodium chloride and 0.3 M tri-sodium citrate
- Antidigoxigenin-AP conjugate antibody stored at 4°C
- CDP-Star (Perkin Elmer) stored at 4°C

3. α -factor synchronization

- Appropriate selective medium
- 40% Raf
- YEP medium (1% yeast extract, 2% peptone, 60 mg/mL adenine, pH 6.5).
- 5 mg/mL α -factor
- 10% sodium azide.
- 40% galactose (Gal)
- 10 mg/mL doxycycline

4. Minichromosome DNA extraction

- NIB (nuclear isolation buffer) 17% glycerol (v/v), 50 mM MOPS, 150 mM AcK, 2 mM Cl_2Mg , 500 mM spermidine, 150 mM spermine stored at 4°C.
- Glass beads (0.5 mm of diameter)
- TEN₅₀₋₅₀₋₁₀₀ (50 mM TRIS, 50 mM EDTA, 100 mM NaCl)
- 30% sarcosyl
- Proteinase K (Roche).
- P-CIA 25:24:1 stored at 4°C
- CIA 24:1 stored at 4°C
- 100% ethanol
- 10 mg/mL RNase A

4. DNA treatments

- Endonuclease nicking enzyme
- Appropriate restriction buffer

5. Two-dimensional agarose gel electrophoresis

- Agarose (Seakem LE; Lonza)
- 5× TBE (Tris-Borate-EDTA) 0.445 M Tris base, 0.445 M boric acid and 0.01 M EDTA
- 10× gel loading buffer: 0.1 % xylene cyanol, 0.1 % bromophenol blue, 30 % glycerol and 10 mM EDTA, pH 8.0

METHODS

1. Yeast transformation

- Transfect competent *S. cerevisiae* cells with monomeric forms of the minichromosomes to study (see Note 1).
- Inoculate and grow the yeast strain in YEP medium containing 2% Raf 16-20 hours at 25°C (OD₆₀₀ has to be >1,5).
- Dilute in 300 mL of YEP medium containing 2% Raf to get a OD₆₀₀ = 0.2-0.3
- Grow the culture at 25°C up to an OD₆₀₀=0.5-0.6
- Centrifuge 50 mL in a 50 mL Falcon tube at room temperature and 3000 rpm for 5 min.
- Throw out the supernatant, wash the cells with 50 mL of sterile cold distilled water and centrifuge again in the same conditions.
- Resuspend the pellet in 3 mL of 1x TE pH 7.5 and transfer 1.5 mL to a 1.5 mL Eppendorf tube.
- Centrifuge in a microfuge at room temperature and maximum speed for 30 seg.
- Carefully resuspend the pellet in 600 µL of 1,1x TE/ 1,1x LiAc.
- Mix in a Falcon tube 300-500 ng of plasmid DNA, 100 µg of denatured salmon sperm DNA and 50 µL of competent cells.

- Add 0.5 mL of 40% PEG/ 1xTE/ 1xLiAc and mix perfectly.
- Incubate the tube at 25°C for 30 min.
- Add 20 µL of DMSO, mix carefully and incubate at 42°C for 15 min with moderate agitation.
- Transfer the volume to a sterile Eppendorf tube and centrifuge at maximum speed for 30 seg.
- Resuspend the pellet in 1 mL of appropriate selective medium containing 2% Raf and transfer the volume to a new transformation Falcon tube.
- Incubate it at 25°C for 1 h and transfer the volume to a sterile Eppendorf tube.
- Centrifuge in a microfuge at room temperature and maximum speed for 30 seg and throw out 700 µL of supernatant.
- Resuspend the pellet in the remaining supernatant and spread the cells on two agar plates containing the appropriate selective medium with 2% Raf (200 µL in one plate and 100 µL in another plate) to select for cells that incorporated the minichromosome
- Incubate at 25°C for 2-3 days.
- Amplify the cells streaking single colonies in a new agar plate containing the appropriate selective medium with 2% Raf to have enough material.

2. Transformation checking

a) DNA fast isolation

- Inoculate and grow one colony in 15 mL of appropriate selective medium containing 2% Raf (as many tubes as colonies you want to check) overnight at 25°C (OD₆₀₀ has to be >3) .
- Harvest the cells by centrifugation at 4°C and 3000 rpm for 10 min.
- Throw out the supernatant and wash the cells with 1 mL of sterile cold distilled water and centrifuge again in the same conditions.
- Transfer the volume to an Eppendorf tube with screw top and centrifuge in a microfuge at maximum speed for 20 seg.
- Resuspend the pellet in 200 µL of breaking buffer and add 0.3 g of glass beads and 200 µL of P-CIA.

- Lyse the cells in a FastPrep®-24 Instrument (x2 cycles at 4°C and 4 m/s for 20 seg waiting 20 seg between cycles) .
- Add 200 µL of TEN₁₀₀ and centrifuge samples in a microfuge at room temperature and maximum speed for 5 min.
- Transfer the supernatant to a fresh Eppendorf tube with screw top.
- Extract the proteins at least twice with pH 8.0-equilibrated P-CIA, and once with CIA
- Precipitate de DNA with 2.5 volumes of cold absolute ethanol overnight at -20°C and resuspend it in ultra-pure water containing 1 mg/mL of RNase A.
- Incubate at 37°C for 1h.

b) Unidimensional agarose gel electrophoresis

- Assemble a gel casting set with a 20-teeth (0.5 × 0.15 cm each) comb and pour a 0.6 % agarose gel in 1× TBE buffer. Let the gel solidify.
- Place the gel into a gel tank and pour 1× TBE buffer to cover the gel. Carefully remove the comb.
- Add 1× gel loading dye to the DNA samples and load the samples into the gel wells. You have to load your samples intact and linearized in order to check the size properly. The controls are the material which was used to transform the yeast (intact and linearized) .
- Run at 0.8 V/cm at room temperature for 15-20 h (depending on the minichromosome size) .
- After electrophoresis transfer the DNA from the gels to an appropriate membrane by Southern blotting.

c) Southern blotting

- To depurinate the DNA prior to transfer, submerge the gels for 15 min in 500 mL of 0.25 M hydrochloric acid with moderate shaking at room temperature.
- Next, set up the blot transfer as follows avoiding the formation of air bubbles: Place three sheets of Whatman 3 MM paper that has been soaked with 0.4 M sodium hydroxide atop of a “bridge” that rests in a shallow reservoir of 0.4 M sodium hydroxide. Place the gel atop of the three soaked sheets of Whatman 3 MM paper.

Roll a sterile pipette over the sandwich to remove all air bubbles that often form between the gel and the paper. Cut a piece of positively charged Zeta-probe blotting membrane to the size of the gel. Pre-soak the membrane in distilled water and place it on top of the gel. Use a pipette to eliminate air bubbles as above. Complete the blot assembly by adding three sheets of Whatman 3 MM paper soaked in 0.4 M sodium hydroxide, a stack of paper towels, a glass plate, and a 200–500 g weight (*see Note 2*)

- Allow the blot transfer overnight in transfer buffer (0.4 M sodium hydroxide) .
- After the transfer, peel the membrane from the gel; rinse it twice in $2\times$ SSC for 5 min.

d) Non-radioactive hybridization

- Label the DNA probes with digoxigenin using the DIG- High Prime kit (Roche) according to manufacture's recommendations.
- Meantime, pre-hybridize the membranes in a 20 mL prehybridization solution ($2\times$ SSPE, 0.5 % Blotto, 1 % SDS, 10 % dextran sulfate and 0.5 mg/mL sonicated and denatured salmon sperm DNA) in hybridization bottles on a rotisserie inside a hybridization oven set at 65°C for 4–6 h.
- Denature the labelled DNA probe by heating it at 95–100°C for 5 min and chill it quickly in an ice bath.
- Add the probe to the hybridization bottles, place the bottles back into the oven and hybridize for 12–16 h.
- Wash the hybridized membranes sequentially with $2\times$ SSC and 0.1 % SDS at room temperature for 5 min twice, and then twice with $0.1\times$ SSC and 0.1 % SDS at 68°C for 15 min.
- Perform the detection with an antidigoxigenin-AP conjugate antibody (Roche) and CDP-Star (Perkin Elmer) according to the instructions provided by the manufacturer.

3. α -factor synchronization

a) In the presence of topo 2

- Inoculate and grow the yeast strain in 20 mL of appropriate selective medium containing 2% of Raf at 25°C until the culture be saturated.
- Diluted in 150 mL of selective medium containing 2% of Raf ($OD_{600}=0.03$) and grow the cells at 25°C until $OD=0.7$ (14-16 h) .

- Transfer the cells to 300 mL of YEP medium with 2% of Raf ($OD_{600}=0.3$) and grow it at 25°C until midlog ($OD_{600}=0.7$) .
- Add 5 $\mu\text{g/mL}$ of α -factor to arrest cells at G1 and incubate them at 25°C until 90% or more have *shmoo* morphology (approximately 3 h) .
- Harvest the cells by centrifugation at room temperature and 4000 rpm for 2 min.
- Wash the cells 4 times with the same volume of YEP medium with 2% Raf to remove the α -factor and start the release.
- Incubate them at 25°C for 80 min. Time 0 was defined as the time of the first wash.
- Add 0.1% of sodium azide to stop the growth and harvest the cells by centrifugation at 4°C and 3500 rpm for 10 min.
- Wash the cells twice with cold sterile distilled water (after this step the cells can be frozen at -80°C).

b) In the absence of topo 2

- To accomplish inhibition of topo 2 start growing top2-td cells as described before until α -factor addition. In this case 100-120 min after α -factor addition (~ 50% of cells have *shmoo* morphology) add 2% galactose to express the degron ligase ubiquitin and incubate at 25°C for 30-45 min more (until ~ 90% of cells have *shmoo* morphology).
- Add 50 $\mu\text{g/mL}$ doxycycline to repress the top2 gen promoter and incubate at 25°C for 30 min.
- Change the culture to the restrictive temperature (37°C) and incubate it for 90 min to induce the degron ligase ubiquitin.
- Harvest cells by centrifugation at room temperature and 4000 rpm for 2 min.
- To start the release wash the cells 4 times with YEP medium with 2% of Gal and 50 $\mu\text{g/mL}$ doxycycline at 37°C.
- Incubate at 37°C for 80 min. Afterwards, continue as described above.

5. Minichromosome DNA extraction

- Resuspend the cells in cold NIB to get a $2.0 \cdot 10^9$ cel/mL final concentration and add the same volume of glass beads.

- Shake the tubes in a vortex shaker at 4°C and maximum speed for 30 seg Afterwards put the tubes on ice for 30 seg. Repeat this step until you get 90% broken yeasts (check it on the microscope).
- Pass the supernatant to a clean tube and wash the glass beads twice using the same NIB volume. Save the washes.
- Join the two washes with the original supernatant.
- Centrifuge at 4°C and 6500 rpm for 20 min.
- Resuspend the pellet in 5 mL of 37°C pre-heated TEN₅₀₋₅₀₋₁₀₀ containing 1.5 % of sarcosyl.
- Add 300 µg/mL proteinase K and mix carefully.
- Incubate at 37°C for 1h and centrifuge at 4°C and 4000 rpm for 5 min.
- Pass the supernatant to a clean tube and centrifuge again in the same conditions.
- Extract the proteins at least three times with pH 8.0-equilibrated P-CIA, and once with CIA.
- Precipitate the DNA with 2.5 volumes of cold absolute ethanol overnight at -20°C and resuspend it in ultra-pure water containing 1 mg/mL of RNase A.
- Incubate at 37°C for 1h.

6. DNA treatments

- Add the endonuclease nicking enzyme and perform the reaction according to the instructions provided by the manufacturer.

7. Two-dimensional agarose gel electrophoresis

- The first dimension is as described in paragraph 2b but using 0.4% agarose concentration gel and running it at 0.4 V/cm for 22-28h (see Note 3).
- Stop the first dimension run.
- Take the gel-casting tray with the gel out of the tank and place it on a clean surface. Cut out each lane from the first dimension and place it on top of another gel casting tray oriented 90° with respect to the first dimension so that the well side of the first dimension slice is on the left side of the second dimension.

- Melt 0.5–1.2% agarose gel in 1× TBE, cool it down to 50–55°C and pour over the slices from the first dimension. Let the gel solidify.
- Place the gel into a gel tank and pour cold 1x TBE to cover the gel.
- Run the second dimension at 0.9–5 V/cm at 4°C for 8–14 h (depending on the minichromosome size) .
- After electrophoresis, transfer the DNA from the gels to an appropriate membrane by Southern blotting and perform non-radioactive hybridization (see 2c and 2d above) .

NOTES

1. As described by Murray and Szostak in 1983 (12), a circular artificial minichromosome needs specific elements to replicate extrachromosomally in an autonomously manner:
 - ARS (autonomously replicating sequence): This element acts as a replication origin and confers minichromosomes the ability to replicate extrachromosomally.
 - CEN (centromere): increases mitotic stability. Without this element minichromosomes are mitotically unstable and are rapidly lost when there is no selective pressure. Moreover, the presence of CEN controls copy number from 20-50 (without CEN) to 1-2 minichromosomes per cell (with CEN) .
 - In addition the minichromosome must bear a selective gene to select those cells that have incorporated the minichromosome after transfection.
 - It should have also a replication origin active in *E. coli* cells and a selective gene to select the transformed cells in *E. coli*.
2. Make sure there is no short-cut between the wick and the paper towels, i.e., they should not touch each other. The capillary force created by the dry towels has to go through the gel only for the DNA to migrate from the gel onto the membrane. A cling film or other wrap is often placed around the gel to prevent short-cuts.
3. Intact circular DNA molecules are analysed in N/N 2D gels as described before (13). Electrophoresis conditions, though, vary significantly depending on the mass of the molecules to be analyzed. Finding the correct electrophoresis conditions for small or large molecules may require some experimentation using molecules of known masses. In our hands, for molecules ~2.5 Kbp the first dimension is usually run in a 0.5 % agarose gel at 1 V/cm for approximately 20–24 h. The second dimension is run in a 1.2 % agarose gel at 5–

6 V/cm for 8–12 h at 4 °C. Molecules larger than 6–8 Kbp must be run under conditions of lower agarose concentration and lower voltage in both dimensions in order to separate different topoisomers successfully. For example, for plasmids of ~10 Kbp the first dimension is usually run in a 0.28 % agarose gel at 0.45 V/cm for approximately ~70 h. The second dimension is run in a 0.58 % agarose gel at 0.9 V/cm for ~90 h at room temperature.

Acknowledgements

This work was sustained by grant BFU2014-56835 from the Spanish Ministerio de Economía y Competitividad to JBS. We acknowledge plasmids and DNA sequence data to Jonathan Baxter and Luis Aragón. The authors also acknowledge Alicia Rodríguez-Bernabé for technical help.

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Legend to Figures

Figure 1: Cartoons illustrating unreplicated and partially replicated molecules. A: Unreplicated. **B:** Partially replicated. **C:** Progression of the replication fork generates positive (+) supercoiling showing left-handed (LH) crossings ahead. **D:** Swiveling of the fork diffuses one LH crossing from the unreplicated to the replicated region, where it adopts the form of two precatenane right-handed (RH) crossings. Parental DNA is colored in blue and green and nascent DNA in red (reproduced from ref. 11 with permission from plosone.org).

Figure 2: Cartoons and immunograms illustrating the different patterns generated by the stereoisomers of undigested circular minichromosomes and linear DNA replication intermediates in 2D gels. A: Linear forms (1.0x and 2.0x) and covalently closed monomers (CCCm) and dimers (CCCd) are depicted in black. CatA (catenanes where both rings are nicked) are depicted in light blue. CatB (catenanes where one ring is nicked and the other covalently closed) are depicted in red. CatC (catenanes where both rings are covalently closed) are depicted in green. KnCatA (nicked-catenanes where one or both rings are knotted) are depicted in yellow. And knotted monomers (Knm) are depicted in black encircled yellow.

B: Synchronized top2-td cells transformed with pYAC_MEM were fixed 80 minutes after their release into the S-phase under permissive (left) or restricted (right) conditions.

Immunograms of 2D gels corresponding to undigested forms of pYAC_MEM are shown on top. The same DNA digested with the nicking enzyme NtBpU10I are shown below. The immunograms are shown together with corresponding diagrammatic interpretations of the most prominent signals to their right (reproduced from ref. 11 with permission from plosone.org).

Figure 1

Figure 2