SUPPLEMENTARY MATERIAL

Supplementary Discussion

The notion that replicating DNA can be unlinked by precatenane removal (Champoux and Been, 1980) is supported by a number of studies in E. coli (recently reviewed in Espeli and Marians, 2004; Schvartzman and Stasiak, 2004) and by our study in Xenopus (Lucas et al., 2001). However, the universality of this mechanism is still open to discussion.

First, a referee of this paper points out that the chiral substrate preferences of the prokaryotic type-2 topoisomerases, gyrase and topo IV, question the precatenane model in bacteria. Gyrase has the unique ability to introduce (-) supercoils but is a poor decatenase. Gyrase action ahead of the forks may be potent enough to maintain a high (-)ΔLk until the unreplicated region becomes too small. Topo IV, on the other hand, removes any remaining (+) catenanes. Initially, topo IV was also suggested to remove (+) precatenanes once gyrase action becomes insufficient to maintain a (-)ΔLk. However, topo IV was recently found to have a chiral preference for the DNA crossing geometry preferentially found in the left-handed superhelix of (+) supercoils and (-) catenanes (Charvin et al., 2003; Crisona et al., 2000; Stone et al., 2003). It was argued that removing (+) supercoils would unlink replicating DNA but removing (-) precatenanes would be harmful as it would increase Lk and cancel the action of gyrase (Schvartzman and Stasiak, 2004). We think this argument does not apply to eukaryotes because (i) they lack the (-) supercoiling activity of gyrase and (ii) eukaryotic topo II appears insensitive to the chirality of DNA crossings in single-molecule experiments (Charvin et al., 2003; Strick et al., 2000).

More recently, yeast topo II was reported to slightly shift the equilibrium distribution of Lk in DNA circles by preferential removal of (-) supercoils (Trigueros et al., 2004). The underlying mechanism does not appear to rely on chiral discrimination of DNA crossings and its eventual implications for precatenane removal remain to be investigated.

Second, the same referee suggests that our observation that topo II acts behind the forks does not necessarily prove the presence of precatenanes. We agree that this observation alone does not tell what topo II is doing behind the forks, but the added fact that topo II inhibition increases the ΔLk of replication intermediates would be difficult to explain otherwise than by inhibition of precatenane removal since no action of topo II was detected ahead of the forks (Lucas et al., 2001).

Finally, one cannot exclude that the use of mutants or drugs to probe topoisomerase function perturbs replication dynamics and causes precatenane formation. One could imagine that replication forks normally are not free to spin about their axis, thus preventing precatenane formation, but that upon topoisomerase inhibition accumulation of (+) supercoils causes replication fork stalling and replisome disassembly, allowing in turn the free diffusion of the (+) supercoils and their conversion to (+) precatenanes before replication fork restart. Support for this model comes from experiments in E. coli showing that PriA, the key protein of the main replication restart pathway, is required for the viability of gyrase subunit GyrB mutants (Grompone et al., 2003). In Xenopus, however, the presumption that topo II inhibition would induce the accumulation of (+) supercoils is difficult to reconcile with the dominant relaxation activity of topo I and the observation that topo II does not detectably act ahead of replication forks (Lucas et al., 2001). Furthermore, no clear equivalent of the PriA replication restart pathway has been found in eukaryotes.

Methods

Drugs, DNA templates, recombinant proteins, antibodies and egg extracts

Stock solutions of ICRF-193 (17.7 mM, Euromedex) and etoposide (10 mM, Sigma) were prepared in dimethylsulfoxide (Sigma) and stored in small aliquots at -20°C. ICRF-193 was used at 150 μM and etoposide at 50 μM in all experiments. Published methods were used to prepare pBR322 DNA (Lucas et al., 2001), M13 mp18 single-stranded DNA (Sambrook and Russell, 2001), demembranated Xenopus sperm nuclei (Murray, 1991), low speed

supernatants (Lucas et al., 2001) and high speed supernatants (Almouzni and Mechali, 1988) of Xenopus eggs. Recombinant geminin-DEL (McGarry and Kirschner, 1998) was produced from an expression plasmid kindly provided by J. Blow and purified on Ni-NTA-Resin (Quiagen) according to the manufacturer's instructions. Recombinant GST-tagged mouse p27Kip1 (Toyoshima and Hunter, 1994) was produced from an expression plasmid kindly provided by J. Walter, purified on glutathion-sepharose 4B (Pharmacia), and dialyzed against PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4).

DNA replication and chromatin assembly

Demembranated sperm nuclei were incubated at 1000 or 5000 nuclei/µl in LSS at 23°C. At 30 min, replicating DNA was labelled for 1 min by addition of 0.5 µCi/µl [α-^{32}P]dATP followed by a chase with 2.5mM dATP and $MgCl_2$. After the indicated chase times, 0.4% NP40, 3mM $CaCl_2$ and 2 u/µl micrococcal nuclease (Roche) were added. Reactions were incubated at 23°C for 0.5-8 min and stopped by diluting in 6 vol of 1% SDS, 40 mM EDTA, 300 mM NaCl. Samples were digested with RNase A and proteinase K, extracted with phenol, precipitated with ethanol, resuspended in 10 mM Tris-Cl pH 8.0, 1 mM EDTA, electrophoresed through 1.5 % agarose gels in the presence of 0.5 µg/ml ethidium bromide, blotted onto Hybon-N+ (Amersham) and directly exposed to a phosphorimager screen. For the experiments in p27Kip1- and geminin-treated extracts, [α-^{32}P]dATP was omitted and the blot was hybridized with a BamHI-digested total Xenopus sperm DNA probe.

M13 single-stranded DNA was incubated at 5 or 15 ng/µl in HSS supplemented with and ATP regenerating system (100 µg/ml creatine phosphokinase (Sigma), 7.5 mM phosphocreatine, 2 mM ATP-$MgCl_2$) and 0.5 µCi/µl [α-^{32}P]dATP for 2-3 h at 23°C. Micrococcal nuclease digestion was as for sperm nuclei except that NP40 was omitted and [nuclease] was 7 u/µl. For two-dimensional chloroquine gel electrophoresis, undigested samples were extracted as above and electrophoresed in 0.66% agarose at 1.75 V.cm^{-1} for 22 h in the presence of 0.6 µg/ml (1st dimension) and 2.0 µg/ml chloroquine (2nd dimension).

Extract immunodepletion and topo II addition

Anti-Xenopus topo IIα antiserum (Luke and Bogenhagen, 1989), or control non-immune rabbit serum (Sigma), were cross-linked to protein A-sepharose fast flow beads (Amersham) according to a standard procedure (Harlow and Lane, 1988). 100µL of HSS was incubated with 100 µL of dried control or anti-topo II cross-linked beads at room temperature for 1h. Extracts were harvested by brief centrifugation through home-made minicolumns and used for M13 replication reaction as described above.

For topo II addition experiments, extracts were added with an 0.4 u/µl recombinant human topo IIα (Amersham), an amount equivalent to 2-3 fold extract's endogenous topo II activity, as judged by a kinetoplast DNA decatenation assay. ICRF-193 (150 µM) fully inhibited the decatenation activity of the complemented extract (data not shown).

Supplementary References

Almouzni G, Mechali M (1988) Assembly of spaced chromatin involvement of ATP and DNA topoisomerase activity. *Embo J* **7**: 4355-65.

Champoux J, Been M. (1980) Topoisomerases and the swivel problem. In Alberts, B. (ed.) *Mechanistic studies of DNA replication and genetic recombination: ICN-UCLA symposia on molecular and cellular biology*. Academic Press, New-York, pp. 809-815.

Charvin G, Bensimon D, Croquette V (2003) Single-molecule study of DNA unlinking by eukaryotic and prokaryotic type-II topoisomerases. *Proc Natl Acad Sci U S A* **100**: 9820-5.

Crisona NJ, Strick TR, Bensimon D, Croquette V, Cozzarelli NR (2000) Preferential relaxation of positively supercoiled DNA by E. coli topoisomerase IV in single-molecule and ensemble measurements. *Genes Dev* **14**: 2881-92.

Espeli O, Marians KJ (2004) Untangling intracellular DNA topology. *Mol Microbiol* **52**: 925-31.

Grompone G, Ehrlich SD, Michel B (2003) Replication restart in gyrB Escherichia coli mutants. *Mol Microbiol* **48**: 845-54.

Harlow E, Lane DP. (1988) *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Lucas I, Germe T, Chevrier-Miller M, Hyrien O (2001) Topoisomerase II can unlink replicating DNA by precatenane removal. *EMBO J.* **20**: 6509-6519.

Luke M, Bogenhagen DF (1989) Quantitation of type II topoisomerase in oocytes and eggs of Xenopus laevis. *Dev Biol* **136**: 459-68.

McGarry TJ, Kirschner MW (1998) Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93**: 1043-53.

Murray AW (1991) Cell cycle extracts. *Methods Cell Biol* **36**: 581-605.

Sambrook J, Russell DW. (2001) *Molecular cloning - a laboratory manual*. CSHL Press, Cold Spring Harbor, New York.

Schvartzman JB, Stasiak A (2004) A topological view of the replicon. *EMBO Rep* **5**: 256-61.

Stone MD, Bryant Z, Crisona NJ, Smith SB, Vologodskii A, Bustamante C, Cozzarelli NR (2003) Chirality sensing by Escherichia coli topoisomerase IV and the mechanism of type II topoisomerases. *Proc Natl Acad Sci U S A* **100**: 8654-9. Epub 2003 Jul 11.

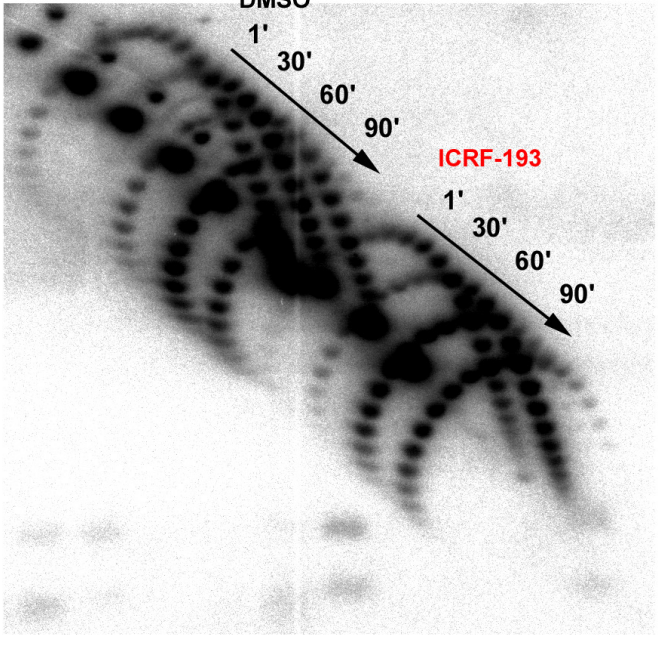
Strick TR, Croquette V, Bensimon D (2000) Single-molecule analysis of DNA uncoiling by a type II topoisomerase. *Nature* **404**: 901-4.

Toyoshima H, Hunter T (1994) p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78**: 67-74.

Trigueros S, Salceda J, Bermudez I, Fernandez X, Roca J (2004) Asymmetric removal of supercoils suggests how topoisomerase II simplifies DNA topology. *J Mol Biol* **335**: 723-31.

Legend to Supplementary Figure

Effect of ICRF-193 on replication-independent chromatin assembly on double-stranded plasmid DNA in HSS. pBR322 DNA was incubated in HSS at 5 ng/µl with 150 µM ICRF-193 or drug solvent alone (DMSO). At the indicated times DNA was extracted and analyzed by 2D chloroquine gel electrophoresis and blot hybridization with a pBR322 DNA probe. ICRF-193 decreased nucleosome density (2 independent experiments) to an extent comparable with replication-coupled chromatin assembly (compare with Figure 3). These results confirm that ICRF-193 can perturb chromatin structure independently of DNA replication.


DMSO
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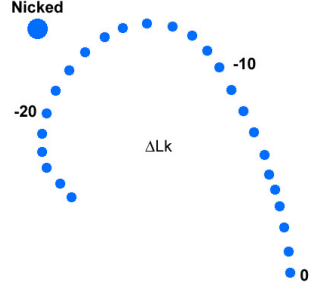
Nicked
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