Chromatin regulates DNA torsional energy via topoisomerase II-mediated relaxation of positive supercoils

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Running title: DNA torsional stress in chromatin

Abstract

Eukaryotic topoisomerases, topo I and topo II, relax the positive and negative DNA torsional stress (TS) generated ahead and behind the transcribing machinery. It is unknown how this DNA relaxation activity is regulated and whether (+) and (-)TS are reduced at similar rates. Here, we used yeast circular minichromosomes to conduct the first comparative analysis of topo I and topo II activities in relaxing chromatin under (+) and (-)TS. We observed that, while topo I relaxed (+) and (-)TS with similar efficiency, topo II was more proficient and relaxed (+)TS more quickly than (-)TS. Accordingly, we found that the relaxation rate of (+)TS by the endogenous topoisomerases largely surpassed that of (-)TS. We propose a model of how distinct conformations of chromatin under (+) and (-)TS may produce this unbalanced relaxation of DNA. We postulate that, while quick relaxation of (+)TS may facilitate the progression of RNA and DNA polymerases, slow relaxation of (-)TS may serve to favor DNA unwinding and other structural transitions at specific regions often required for genomic transactions.

Keywords not appearing in the title:
supercoiling/gyrase/nucleosome/transcription/yeast

Synopsis

A comparative analysis of topo I and topo II activities in native chromatin reveals that twin domains of (+) and (-) DNA torsional stress, which are simultaneously generated during DNA transcription, are not relaxed at the same rate.

- Chromatin delays the relaxation of both (+) and (-) DNA torsional stress by topo I.
- Chromatin promotes quick relaxation of (+) DNA supercoils by topo II.
- Unbalanced relaxation of (+) supercoils may facilitate DNA unwinding in eukaryotic chromatin.

Introduction

In eubacteria, such as *Escherichia coli*, the free energy of negative DNA supercoiling is required to initiate chromosome replication, modulate gene expression and shape the nucleoid architecture (Hatfield and Benham, 2002; Travers and Muskhelishvili, 2005). Unconstrained (-) supercoiling or, more precisely, (-) torsional stress (TS) is sustained by DNA gyrase, a type-2 topoisomerase that reduces the linking number (*Lk*) of the DNA double helix (Gellert et al, 1976). Remarkably, a DNA supercoiling enzyme analogous to DNA gyrase is not present in eukaryotic cells (Corbett and Berger, 2004; Chen et al, 2013). In eukaryotic chromatin, (-) supercoiling of DNA is mostly constrained by the periodic folding of DNA into nucleosomes (Prunell, 1998). In this landscape, cellular topoisomerases (topo I and topo II) relax the TS that is generated by DNA tracking motors (i.e. RNA and DNA polymerases, DNA helicases) and other processes that change DNA topology (i.e. nucleosome assembly-disassembly) (Roca 2011). During DNA transcription, rapid rotation of the double helix relative to the RNA polymerase is challenged by the viscous drag of intracellular chromatin (Nelson, 1999). Consequently, (+) and (-)TS are generated at the same rate ahead and behind the transcribing complex, respectively (Liu and Wang, 1987; Giaever and Wang, 1988). Likewise, (+)TS also occurs in front of DNA replications forks. TS can dissipate near the telomeres but it cannot diffuse from the internal regions of linear chromosomes (Joshi et al, 2010; Kegel et al, 2011). Thus, the relaxation of TS by cellular topoisomerases is required for the correct progression of DNA transcription and replication (Wang, 2002). However, it is unclear whether cellular topoisomerases play more specific roles in tuning TS across eukaryotic chromatin.

Numerous observations indicate a functional redundancy of topo I and topo II in the relaxation of TS. DNA replication forks stall when both enzymes are defective but can progress at normal rates in the presence of either topo I or topo II (Kim and Wang, 1989; Bermejo et al, 2007). The relaxation of (+)TS by either topo I or topo II is sufficient for normal advancement of RNA polymerases (Mondal and Parvin, 2001; Mondal et al, 2003; Garcia-Rubio and Aguilera, 2012). Accordingly, yeast Δ*top1* mutants are viable and show minor alterations of gene expression (Brill et al, 1987; Lotito et al, 2008). Likewise, although topo II is essential for chromosome segregation, its inactivation does not preclude RNA synthesis in yeast (Bermejo et al, 2009; Durand Dubief et al, 2010). Only in yeast *Δtop1 top2-ts* double mutants global RNA synthesis is decreased (Brill et al, 1987, Schultz et al, 1992, Sperling et al, 2011; Pedersen et al, 2012). However, other studies have described that the DNA relaxation activities of topo I and topo II are not always interchangeable or able to compensate each other. Circular minichromosomes with DNA under (+)TS are relaxed more efficiently by topo II than by topo I in comparison to naked DNA plasmids (Salceda et al, 2006). Accordingly, topo II rather than

topo I is required in yeast to relax the (+)TS that stalls Pol II during the transcription of long genes (Joshi et al, 2012). Likewise, topo II is preferentially localized near long genes and required for their proper expression in mammalian cells (King et al, 2013; Thakurela et al, 2013). Specific roles of cellular topoisomerases in DNA relaxation have also been observed at the ribosomal genes, in which twin domains of (-) and (+)TS generated during Pol I transcription are preferentially relaxed by the activities of topo I and topo II, respectively (El Hage et al, 2010; French et al, 2011).

The distinct efficiency or specific preferences of topo I and topo II to relax intracellular DNA have been explained in terms of the interplay between chromatin structure and topoisomerase mechanisms (Salceda et al, 2006). Topo I temporarily cleaves one strand of the duplex and permits one free end to rotate in either direction around the uncleaved strand (Stewart et al, 1998; Krogh and Shuman, 2000). As this "strand rotation" mechanism does not require an energetic cofactor, DNA torque and friction drive integral rotations until the relaxation of (+) or (-)TS is completed (Koster et al, 2005). Accordingly, topo I should be effective in chromatin configurations that facilitate axial rotation of DNA. Topo II produces instead a transient double-strand break at one DNA segment, through which it passes another segment of duplex DNA in an ATP-dependent manner (Wang, 1998). This “cross-over inversion” removes the (+) and (-) DNA supercoils that may occur when DNA is under (+) and (-)TS. Accordingly, topo II should be effective in chromatin configurations that favor supercoil extrusion or juxtaposition of DNA segments. These premises explained why topo II is more proficient than topo I in relaxing chromatin under (+)TS (Salceda et al, 2006). In this regard, it remains to be explored whether the same happens when chromatin is under (-)TS. This comparison is important to address a fundamental issue, namely whether cellular topoisomerases are able to remove with similar efficiency the twin domains of (+) and (-)TS generated during *in vivo* DNA transcription regardless of other factors that may regulate the localization and catalytic activity of these enzymes.

To tackle the above questions, here we revisited an earlier study by Brill and Sternglanz (1988), which reported that transcriptionally active circular minichromosomes in yeast Δ*top1* and Δ*top1 top2-ts* mutants undergo a large reduction of *Lk*. Our experiments corroborate this observation and show that the DNA superhelical density of the minichromosomes falls from -0.05 (typical of chromatin) to values as low as -0.12; and that this change results from an accumulation of (-)TS that preserves the nucleosomal organization of DNA. Therefore, we used these minichromosomes to conduct the first comparative analysis of topo I and topo II activities in relaxing (+) and (-)TS in native chromatin. We show that, while topo I relaxes (-) and (+)TS slowly and at similar speed, topo II relaxes (+)TS more quickly than (-)TS. This imbalance is consistent with the accumulation of (-)TS observed in Δ*top1*mutants and altogether indicates that the intracellular relaxation rate of (+)TS largely surpasses that of (-)TS. We present a model

to explain how distinct configurations of chromatin under (+) and (-)TS affect the DNA relaxation capacity of cellular topoisomerases. We postulate that chromatin promotes the rapid relaxation of (+)TS in order to facilitate the progression of RNA and DNA polymerases, while it delays the relaxation of (-)TS in order to keep the DNA torsional energy that is often required for genome transactions.

Results

Inactivation of topo I and topo II reduces the DNA linking number of yeast circular minichromosomes

Eukaryotic chromatin constraints on average about one negative helical turn of DNA per nucleosome. Consequently, the average linking number (*Lk*) of chromatinized DNA is smaller than of freely relaxed DNA (Lk^o) and this *Lk* difference ($\Delta Lk = Lk - Lk^o$) roughly correlates to the number of assembled nucleosomes. In this regard, we observed that the *Lk* values of some circular minichromosomes in yeast topoisomerase mutants were markedly reduced. This phenomenon is shown in Figure 1A, which displays by two-dimensional DNA gel electrophoresis the *Lk* distributions of various plasmids extracted from Δ*top1 top2-4* double-mutants before and after inactivation of the thermo-sensitive topo II enzyme (1 h at 37ºC). In these gels, *Lk* topoisomers distribute along an arch, where *Lk* values increased clockwise (+) and decreased anti-clockwise (-). In the case of multi-copy plasmids YEpTA1, YEp13 and 2-micron, the *Lk* reduction occurred after the cells were shifted to 37ºC. In the case of single-copy plasmids YCpTA1 and YCp50, the decrease in the *Lk* was incipient at 28ºC and affected nearly all the plasmid molecules when the cells were shifted to 37ºC (Figure 1A and 1D). While the bulk of single-copy plasmids underwent *Lk* reduction, only subfractions of the multi-copy plasmids were affected, even after extending the topo II inactivation time (Figure 1B). Regardless of the plasmid copy-number, this reduction in *Lk* did not occur in the parental *TOP1TOP2* strain or in the Δ*top1* or *top2-4* single-mutants (Figure 1C and 1D). Therefore, this change in DNA topology was caused by a defect in topoisomerase activity and was not related to the thermal shift.

DNA transcription causes the *Lk* reduction in yeast topoisomerase mutants

To examine whether the *Lk* reduction was associated with DNA transcription, replication or centromere activities we constructed plasmid YCp321, which has two 58-bp direct repeats that are recognized by the site-specific recombinase from *Zygosaccharomyces rouxii* (Matsuzaki et

al, 1990). Site-specific recombination splits YCp321 into one circle containing the CEN4 and ARS1 elements and another circle containing the *URA3* gene (Figure 2A). These two circles were produced in Δ*top1 top2-4* yeast mutants, which contained YCp321 and the recombinase expression plasmid pHM53, by shifting the cells from glucose- to galactose-containing media for 6 hours. Following topo II inactivation, *Lk* reduction occurred in the circle containing the URA3 gene but not in that holding the CEN and ARS elements (Figure 2A).

To further assess the dependence of the *Lk* reduction on transcription activity, we constructed the plasmid YCp50 *pGAL1:LacZ,* in which the *E.coli LacZ* gene was under the galactose-inducible *GAL1* yeast promoter (*pGAL1*). We introduced the plasmid in Δ*top1 top2-4* yeast mutants and induced high transcription of *LacZ* by shifting the cells from glucose- to galactose-containing media. Next, we inactivated topo II and checked the plasmid topology at a range of time points (Figure 2B). In the cells kept in glucose medium, the reduction in *Lk* occurred after topo II inactivation since this plasmid contained the *URA3* gene. However, in those cells shifted to galactose medium, the *Lk* value of virtually all the plasmid molecules decreased before the inactivation of topo II. This *Lk* reduction was reverted when cells were shifted back to glucose-containing media that inhibits *pGAL1* (Figure 2C, left). This correlation between *Lk* reduction and induced transcription did not occur in the parental plasmid YCp50 that lacks *pGAL1* (Figure 2C, right). We next examined the topology of YCp50 *pGAL1:LacZ* in the parental *TOP1 TOP2* strain or in the Δ*top1* or *top2-4* single-mutants (Figure 2D). No significant changes occurred in *TOP1 TOP2* cells or in the *TOP1 top2-4* single-mutant, even after topo II inactivation. However, the plasmid in the Δ*top1 TOP2* single-mutant presented an *Lk* reduction resembling that detected in the Δ*top1 top2-4* double mutant. Therefore, when DNA was highly transcribed, the *Lk* reduction occurred even in presence of normal topo II activity.

***Lk* reduction in Δ*top1 top2-ts* yeast cells doubles the typical DNA supercoiling density of chromatin and it is not constrained**

Figure 3A compares the *Lk* distributions of YCpTRP1 extracted from *TOP1 TOP2* and Δ*top1 top2-4* yeast cells after 1 h incubation at 37ºC and of the same plasmid relaxed *in vitro* with topo I at 37ºC. The *Lk* difference between the relaxed plasmid (Lk^o) and the chromatinized plasmid in *TOP1 TOP2* cells was about -22. As YCpTRP1 is 4512 bp in length, its Lk^o is about 428 (4512 bp/10.5). Therefore, the specific *Lk* difference or DNA supercoiling density ($\sigma = \Delta Lk/Lk^o$) of YCpTRP1 in *TOP1 TOP2* cells was roughly -0.05 (-22/428). To calculate σ of YCpTRP1 in Δ*top1 top2-4* cells, we used higher chloroquine concentrations in the electrophoresis such that individual topoisomers within the population of reduced *Lk* values could be counted. YCpTRP1 from Δ*top1 top2-4* cells presented a broad *Lk* distribution with

Δ*Lk* values ranging from about -22 down to -52 (Figure 3B). Thus, the DNA supercoiling density of the minichromosome reached levels as low as -0.12 (-52/428).

We next addressed whether this gain of negative DNA supercoiling density produced in Δ*top1 top2-4* mutants was stabilized by some structure (i.e. DNA unwinding proteins, DNA-RNA interactions, non-B-DNA conformations) or could instead be relaxed as free (-)TS. For this purpose, we solubilized the native YCpTRP1 minichromosomes from lysates of *TOP1 TOP2* and Δ*top1 top2-4* cells after 1 h incubation at 37ºC. The minichromosomes were then incubated with an excess of topo I in the presence of another supercoiled plasmid that served as a DNA relaxation control. The *Lk* distribution of the YCpTRP1 minichromosome solubilized from *TOP1TOP2* cells did not change after incubation with topo I, whereas the control plasmid included in the reaction was relaxed (Figure 3C). The control plasmid was partially relaxed even without the addition of topo I, most likely by the endogenous topoisomerase activity present in the *TOP1TOP2* lysate. These results confirmed that the negative DNA supercoiling density of YCpTRP1 (σ~-0.05) was stabilized by nucleosomes. In contrast, the incubation with topo I of the minichromosome solubilized from Δ*top1 top2-4* cells (σ down to -0.12) produced an *Lk* distribution very similar to that observed in *TOP1 TOP2* cells (Figure 3C). Therefore, the gain of negative DNA supercoiling density produced in Δ*top1 top2-4* mutants was not stabilized. Otherwise, the *Lk* distribution would had remained unaltered while the control plasmid was relaxed. Therefore, the *Lk* reduction stand as free (-)TS, and seemingly this (-)TS did not disrupt the nucleosomal organization of DNA, since the minichromosome recovered its typical DNA topology (σ~-0.05) upon relaxation.

Topoisomerase II relaxes chromatin under (+)TS faster than under (-)TS

Our previous studies showed that yeast minichromosomes with DNA under (+)TS are efficiently relaxed by topo II but not by topo I (Salceda et al, 2006). The accumulation of (-)TS in yeast minichromosomes reported here provided the opportunity to conduct the first comparative analysis of topo I and topo II activities in relaxing (+)TS and (-)TS in native chromatin. Thus, we solubilized the YCpTRP1 minichromosome under (-)TS (σ -0.05 to -0.12) from Δ*top1 top2-4* yeast mutants. Likewise, we solubilized the same minichromosome under (+)TS (σ >+0.04) from Δ*top1 top2-4* yeast mutants constitutively expressing the *E.coli* topoisomerase I. As previously reported, (+)TS accumulates in this condition because the bacterial topoisomerase selectively relaxes (-)TS (untwisted DNA regions) and no other activity has the capacity to relax (+)TS after topo II inactivation (Giaever and Wang, 1988). We incubated equivalent amounts of the minichromosomes under (+) and (-)TS with purified yeast topo I and topo II enzymes and compared their relaxation rates (Figure 4). Both topoisomerases

relaxed the (+) and (-)TS of the minichromosomes and produced final *Lk* distributions of DNA supercoiling density of about -0.05. This outcome corroborated that native nucleosomes were not evicted by (+) or (-)TS. However, the relative relaxation rates produced by topo I and topo II were distinct. While the former relaxed the minichromosomes under (+) and (-)TS at a similar speed, the latter relaxed those under (+)TS over 3-fold faster than those under (-)TS (Figure 4).

The capacity of endogenous topoisomerases to relax chromatin under (+)TS largely surpasses that under (-)TS

The accumulation of (-)TS in yeast minichromosomes reported here suggested that the capacity of cellular topoisomerases to relax chromatinized DNA under (+) and (-)TS is not equal. Thus, we examined whether the unbalanced relaxation of (+) and (-)TS observed with the purified topoisomerases is reflected in the endogenous DNA relaxation activity of yeast cells. For this purpose, we prepared YCpTRP1 minichromosomes under (+) and (-)TS as described above and incubated them with fresh lysates of *TOP1 TOP2* yeast cells, either in the presence or absence of ATP (Figure 5). As we showed in previous studies, incubation in the absence of ATP allowed the activity of topo I, whereas incubation in the presence of ATP allowed the combined activity of topo I and topo II (Salceda et al, 2006). Endogenous topo I activity relaxed the minichromosomes under (+) and (-)TS with comparable efficiency. Upon addition of ATP, both forms of the minichromosome were relaxed more quickly, thus reflecting the contribution of endogenous topo II. However, while the relaxation rate improved by two fold in the case of (-)TS, it increased by nearly six fold for (+)TS (Figure 5). These results were consistent with the relaxation efficiencies observed with the purified enzymes and indicated that the cellular dosage of topo II provides more specific activity than the dispensable topo I to relax chromatinized DNA in yeast. Therefore, the global relaxation rate of (+)TS affected by the endogenous topoisomerases largely surpassed that of (-)TS.

Discussion

Our study corroborates the earlier observation of Brill and Sternglanz (1988) that transcriptionally active circular minichromosomes in yeast Δ*top1* mutants undergo a large reduction in their DNA *Lk* value. Accordingly, the *Lk* reduction affects the bulk of single-copy minichromosomes and only subfractions of multi-copy ones, in which the recruitment of transcription machinery is limited. In this regard, we show that this alteration does not occur in

transcriptionally inactive DNA circles and that it is enhanced in highly transcribed ones. We demonstrate that this *Lk* reduction changes the typical supercoiling density of chromatinized DNA (σ -0.05), reducing it to values twice as low (σ -0.12). Remarkably, this gain in supercoiling density does not disrupt the nucleosomal organization of DNA since the minichromosomes recover their normal DNA topology (σ -0.05) upon *in vitro* relaxation by topoisomerases. These findings provided thus the opportunity to investigate the interplay between (-)TS, chromatin structure and topoisomerase activities.

Given that eukaryotic cells do not have gyrase-like activity, the accumulation of (-)TS in yeast Δ*top1* mutants is possibly the result of an unbalanced relaxation of (+) and (-)TS generated during DNA transcription. This imbalance could be explained in two ways. One is that topo I, not topo II, functions as the main relaxase of (-)TS. The *Lk* reduction therefore occurs in the absence of topo I because topo II or some other activity in *top2-ts* mutants selectively removes (+)TS. This scenario, however, is not consistent with the accumulation of (+)TS observed in Δ*top1 top2-ts* double-mutants that express *E.coli* topoisomerase I (Giaever and Wang, 1988). It appears that upon inactivation of topo II in Δ*top1 top2-ts* double-mutants, no other cellular activity is able to relax (+)TS. In addition, it is unlikely that intracellular topo I relaxes the (-) but not the (+)TS. In such a scenario, (+)TS would accumulate in the *TOP1 top2-ts* mutants, and we did not observe this alteration even in highly transcribed minichromosomes. The second explanation for the *Lk* reduction is that intracellular topo II removes (+)TS faster than (-)TS, whereas topo I relaxes both indistinctively. Thus a reduction in *Lk* occurs in Δ*top1 top2-ts* mutants because their low topo II activity becomes limiting to remove (-) but not (+)TS. Likewise, when the generation rate of (+) and (-)TS markedly increases during high transcription activity, a reduction in *Lk* also occurs in Δ*top1 TOP2* mutants because even normal topo II activity is limiting to remove high levels of (-)TS. This *in vivo* scenario fits with the relaxation rates of yeast minichromosomes that we observed *in vitro*. Topo II relaxes chromatin under (+)TS faster than under (-)TS, while topo I removes (+) and (-)TS equally and not as quickly as topo II. Since the endogenous specific activity of topo II to relax nucleosomal DNA is higher than that of topo I (Salceda et al, 2006), these results also clarify why topo I is not essential for yeast viability. Endogenous topo II alone suffices to relax the (+) and (-)TS generated during normal genome transactions, although it relaxes (+)TS more quickly than (-)TS.

The interplay of chromatin conformation under TS and DNA topoisomerase mechanisms

Topo I does not have an intrinsic preference to relax (+) and (-)TS on naked DNA. Single-molecule experiments showed that the DNA relaxation rate by topo I depends on net torque irrespective of the direction of strand rotation (Koster et al, 2005; 2007). Our results indicate

that this property is preserved in chromatinized DNA, although the relaxation rate is about 10 times slower than naked DNA (Salceda et al, 2006). Therefore, topo I activity is probably delayed by the rotational drag of chromatinized DNA and this drag may be comparable under (+) and (-)TS. Like topo I, yeast topo II does not have an intrinsic preference to remove (+) and (-)TS on naked DNA (Roca and Wang, 1996; Charvin et al, 2003; Salceda et al, 2006). Therefore, the unbalanced relaxation of TS reported here reveals that while chromatin under (+)TS produces conformations suitable for the DNA cross-inversion mechanism of topo II, (-)TS produces conformations less appropriate for this activity. These inferences are consistent with the response of chromatin fibers to the axial rotation of DNA observed *in vitro* (Bancaud et al, 2006; Lavelle et al, 2010). Rotations that produce (+)TS markedly shorten the chromatin fiber and induce nucleosome-reversome transitions (Bancaud et al, 2007). Both deformations imply an increase in DNA writhe (supercoiling), in which newly formed (+) DNA crossovers are substrates for topo II activity. A quick transition from a buffering (partial unwrapping of nucleosomal DNA) to a supercoiling (nucleation of positive DNA crossovers) regime was postulated to explain why topo II and not topo I is required for the production of long transcripts *in vivo* (Joshi et al, 2012).

In contrast to (+)TS, the induction of (-)TS by axial rotation of chromatinized DNA does not produce a comparable shortening effect (Bancaud et al, 2006; Lavelle et al, 2010). Consequently, chromatin is likely to accommodate (-)TS by untwisting DNA rather than by nucleating more (-) supercoils than those possibly configured by the left-handed wrapping of nucleosomal DNA. In agreement with our results and recent *in vivo* studies (Teves and Henikoff 2013), nucleosomes are not evicted by (-)TS. Our results indicate that the configuration of chromatin under (-)TS provides a poor substrate for topo II activity, thus implying a scarcity of newly formed (-) DNA crossovers. We therefore postulate that chromatin under (-)TS deforms mainly by tightening the left-handed wrapping of nucleosomal DNA and by unwinding the duplex at the linker regions. These deformations suffice to reach σ values < -0.12, as reported here for yeast minichromosomes.

The model depicted in Figure 6 shows the plausible conformations of chromatin under (+) and (-)TS, and their interplay with the topo I and topo II mechanisms. These conformations, which explain why minichromosomes under (+)TS are relaxed more quickly than under (-)TS, may occur thus at the chromatin regions downstream and upstream the transcribing complexes. Accordingly, while DNA transcription generates (+) and (-)TS at the same rate, cellular topoisomerases reduce more quickly the (+)TS. Consequently, (-)TS persists longer than (+)TS. In normal conditions, this gain of (-)TS may be transient and restricted to specific regions. However, when the generation rate of TS is increased and/or the topoisomerase activity is deficient, an accumulation of (-)TS becomes evident, as shown in the present study.

Persistence of negative DNA torsional stress in eukaryotic cells

The over-winding of DNA produced by (+)TS hinders DNA transcription and replication (Joshi et al, 2010; Roca, 2011). Efficient relaxation of (+)TS may have thus been optimized in eukaryotic chromatin to ensure proper progression of RNA and DNA polymerases. Conversely, the relaxation of (-)TS may be not as crucial since this constraint does not hamper RNA or DNA synthesis. Although excessive (-)TS can cause hyper-recombination (Trigueros and Roca, 2001; 2002), the maintenance of (-)TS may be necessary at specific regulatory regions in order to facilitate DNA bending or unwinding. This condition is well-known in eubacteria, where (-)TS sustained by DNA gyrase is essential for the regulation of gene expression, the initiation of DNA replication and the spatial folding of DNA (Hatfield and Benham, 2002; Travers and Muskhelishvili, 2005). Similar functions may occur in eukaryotic cells, as (-)TS favors the interaction of DNA with transcription factors (Mizutani et al, 1991a; Parvin and Sharp, 1993) and RNA polymerases (Tabuchi and Hirose, 1988; Mizutani et al, 1991b; Schultz et al, 1992). *In vivo* studies showed that (-)TS drives structural transitions of regulatory DNA sequences (Kouzine and Levens, 2007) and triggers functional responses of some gene loci in yeast and human cells (Kouzine et al, 2008; Brooks and Hurley, 2009).

Stably maintained (-)TS has been observed in specific regions of eukaryotic chromosomes (Jupe et al, 1993; Ljungmanand Hanawalt, 1995; Kramer and Sinden, 1997; Matsumoto and Hirose, 2004). Recent genome-wide analyses of DNA topology have revealed that (-)TS persists across specific chromosomal domains and around the transcription start sites of many genes in *S. cereviasiae* (Bermudez et al, 2010; Kouzine et al, 2013; Naughton et al, 2013). Since eukaryotic cells do not have a gyrase-like enzyme, we propose that persistent levels of (-)TS are achieved via the unbalanced relaxation of (+) and (-)TS. Chromatin architecture and its epigenetic modifications are likely to determine how DNA torsional energy is buffered, dissipated or confined within topological domains (Gilbert and Allan, 2014). In the light of our findings, we propose that chromatin structure also determines the extent to which DNA torsional energy is kept or removed by cellular topoisomerases.

Materials and Methods

Strains, plasmids and enzymes

S. cerevisiae strains *TOP1 TOP2* (JCW25), *TOP1 top2-4* (JCW26), *Δtop1 TOP2* (JCW27) and *Δtop1 top2-4* (JCW28) are derivatives of FY251 (S288C genetic background) and have previously been described (Trigueros and Roca, 2002). Schemes of plasmids YEpTA1 (4.5 Kb),

YCpTA1 (4.5 Kb), YCp50 (7.9 Kb), YEp13 (10.7 Kb), YCp50 *pGAL1:LacZ* and YCp321 are illustrated in the figures. YCp321 contains two 58-bp direct repeats recognized by the site-specific recombinase from *Z. rouxii* (Matsuzaki et al, 1990). pHM53 carries the *Z.rouxii* recombinase gene under the galactose-inducible GAL1 promoter of *S. cerevisiae* (Roca et al, 1992). All plasmids were introduced in yeast using the lithium acetate method (Ito et al, 1983). Topo I of vaccinia virus was purified from *E. coli* cells harboring the expression clone pET11vvtop1 (Shuman et al, 1988). Topo I of *S. cerevisiae* was purified from yeast cells harboring the expression clone YCpGAL-TOP1 (Bjornsti and Fertala, 1999). Topo II of *S. cerevisiae* was purified from yeast cells carrying the expression clone YEpTOP2GAL1 (Worland and Wang, 1989).

Yeast growth and DNA extraction

Yeast cells were grown at 28ºC in synthetic selective media. Thermal inactivation of topo II was done during exponential growth (OD ~0.8) by shifting cell cultures to 37ºC. Cells from a 50-ml culture were harvested and washed in Tris-HCl 10 mM (pH 8) EDTA 1 mM, at 4ºC. Cell pellets were suspended in 300 µl water, 300 µl phenol and 300 µl glass-beads (425-600 µm Sigma), and disrupted in FastPrep apparatus (10 sec at power 5). The aqueous fractions were extracted once more by phenol and DNA was recovered by EtOH precipitation.

Solubilization of yeast circular minichromosomes

Yeast cells from 100-ml cultures were harvested and washed in Tris-HCl 10 mM (pH 8) EDTA 1 mM at 4ºC. Cell pellets were suspended at 4ºC in 1 ml of buffer L (Tris-HCl 10 mM pH 8.0, EDTA 1 mM, EGTA 1 mM, NaCl 150 mM, DTT 1mM, Triton-X100 0.1%, pepstatin 1µg/ml, leupeptin 1µg/ml, PMSF 1 mM). About 1 ml of glass beads was added, and the suspension was stirred during 30 seconds at 4ºC for six times. Supernatants were recovered after two successive centrifugations (20,000 x *g* at 4ºC) and then loaded on a 500-µl Sephacryl S-300 column equilibrated with buffer L at 4ºC. Yeast circular minichromosomes were eluted in the first filtration volume. Eluted minichromosomes were supplemented with an excess (1 mg/ml) of a negatively supercoiled plasmid (2 kb) that served as internal control for topoisomerase activity.

Topoisomerase reactions

Mixtures containing yeast minichromosomes and control plasmids were adjusted to 8 mM $MgCl_2$ and 1 mM ATP (when indicated), pre-incubated at 30ºC for 5 min, and then supplemented with catalytic amounts of topoisomerases. Where indicated, mixtures were supplemented instead with serial dilutions of supernatants obtained after glass bead disruption of *TOP1 TOP2* (JCW25) yeast cells, as described above. Following incubations at 30ºC, reactions were quenched at the indicated times by adding one volume of buffer K (EDTA 40

mM, SDS 1%, proteinase K, RNAse A). Following 1 h of incubation at 60ºC, samples were extracted by phenol, and DNA was recovered by EtOH precipitation.

DNA electrophoresis and topology analysis

Agarose concentration (0.6 to 1 %) was adjusted according to the plasmid size. Electrophoreses were carried out at 25ºC in TBE buffer plus 0.6 µg/ml of chloroquine at 50 V for 14 h in the first dimension (top to bottom), and TBE buffer plus 3 µg/ml of chloroquine at 60 V for 8 h in the second (left to right). DNA was blot-transferred to a nylon membrane and probed with ^{32}P-labeled DNA obtained by random priming. *Lk* distributions were analyzed by quantifying the amount of topoisomer populations displayed by phosphorimaging the probed gel-blots. DNA supercoiling density (σ) was calculated with $\sigma = \Delta Lk/Lk^0$ (Wang et al, 1982). ΔLk was determined in the 2D gel images by counting the number of *Lk* topoisomers spanning from the center of the interrogated *Lk* distribution to the center of the *Lk* distribution obtained by relaxing the naked DNA circle *in vitro* (Lk^0). Lk^0 was calculated with N/h^0, where N is the DNA circle size (in bp) and h^0 (10.5 bp/turn) the most probable helical repeat of DNA in the relaxation conditions used (Horowitz and Wang, 1984).

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Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

XF and JR conceived the research, designed experiments and analysed data. XF, OD-I and BM-G prepared materials and conducted experiments. JR wrote the manuscript.

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Figures

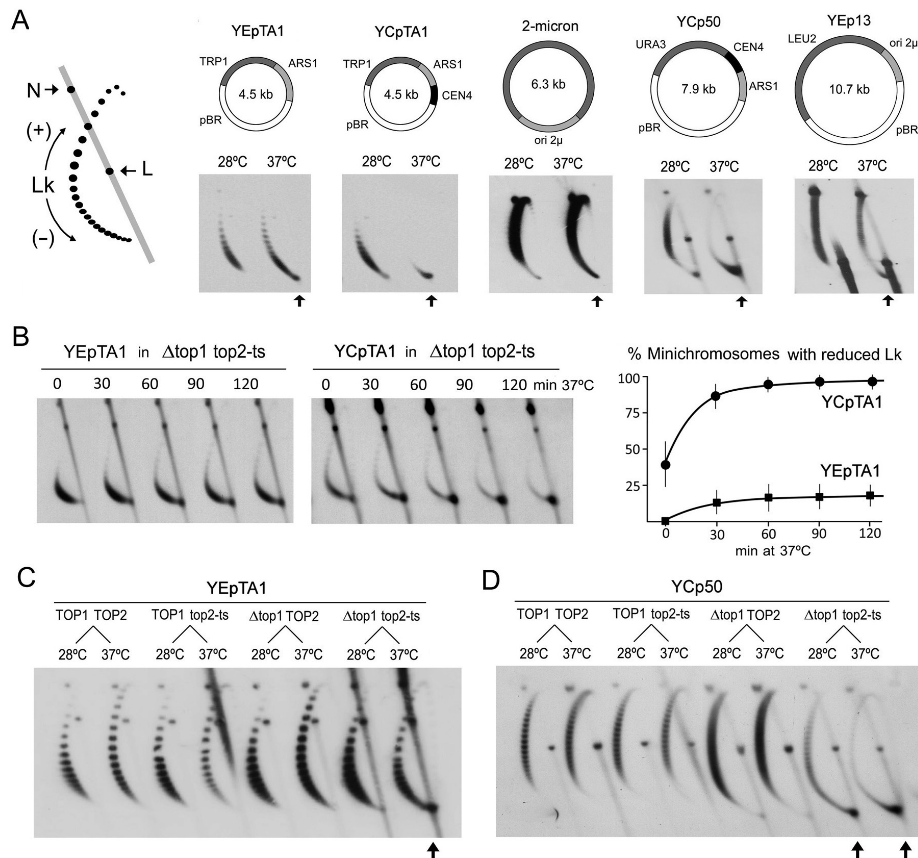

Figure 1. Reduction of DNA linking number of circular minichromosomes in yeast topoisomerase mutants. A) The 2D diagram (left) shows the relative positions of nicked (N), linear (L), and *Lk* distribution of supercoiled DNA topoisomers (Lk). The 2D gel-blots show the *Lk* distributions of plasmids YEpTA1, YCpTA1, 2-micron, YCp50 and YEp13 hosted in yeast Δ*top1 top2-4* double-mutants before (28ºC) and after thermal inactivation of topo II (1 h at 37ºC). Arrows indicate the DNA populations with reduced *Lk*. Plasmid schemes show the position of their functional elements. B) The 2D gel-blots show the *Lk* distributions of YEpTA1 and YCpTA1 at different time points after thermal inactivation of topo II in the yeast Δ*top1 top2-4* double mutant. The graph (rigth) plots the mean and SD of the plasmid fractions with reduced *Lk* in three experiments. C-D) The 2D gel-blots compare the *Lk* distributions of YEpTA1 and YCp50 hosted in the parental *TOP1TOP2* strain and its *TOP1 top2-4,* Δ*top1 TOP2* and Δ*top1 top2-4* derivatives. Cells were sampled before (28ºC) and after thermal inactivation of topo II (1 h at 37ºC).

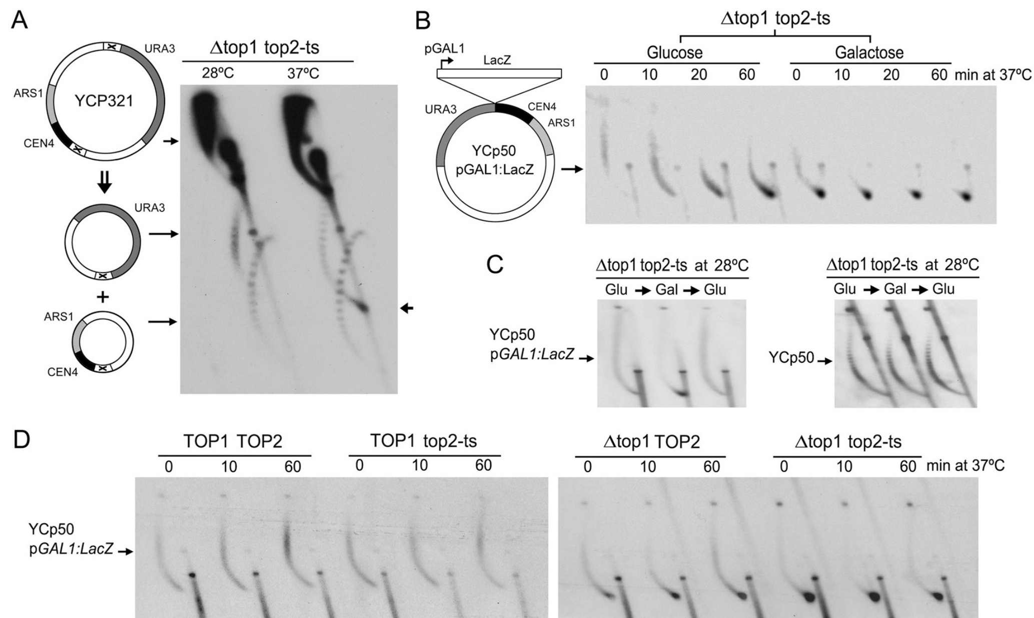

Figure 2. Dependence of the *Lk* reduction on DNA transcription activity. A) The 2D gel-blot shows the topology of YCp321 and its two site-specific recombination circles in yeast Δ*top1 top2-4* double mutants before (28ºC) and after thermal inactivation of topo II (1 h at 37ºC). The arrow in the right indicates the *Lk* reduction in the circle containing the *URA3* gene. B) The 2D gel-blot shows the topology of YCp50 *pGAL1:LacZ* in yeast Δ*top1 top2-4* double-mutants after thermal inactivation of topo II for the indicated times (min). The experiment compares cells that grew in selective media containing 2% glucose and cells that were shifted to 2% galactose-containing media for 3 h to activate the transcription of *LacZ*. C) The 2D gel-blots compare the topology of YCp50*pGAL1:LacZ* and YCp50 in Δ*top1 top2-4* cells after the induction and repression of *pGAL1* at permissive temperature (28ºC). Cells were sampled in media containing 2% glucose, shifted to 2% galactose-containing media for 3 h and shifted back to 2% glucose-containing media for 2 h. D) Experiment conducted as in B, but comparing yeast strains *TOP1 TOP2*, *TOP1 top2-4,* Δ*top1 TOP2* and Δ*top1 top2-4*. The 2D gel-blots show the topology of YCp50 *pGAL1:LacZ* after shifting the cells to the galactose-containing media.

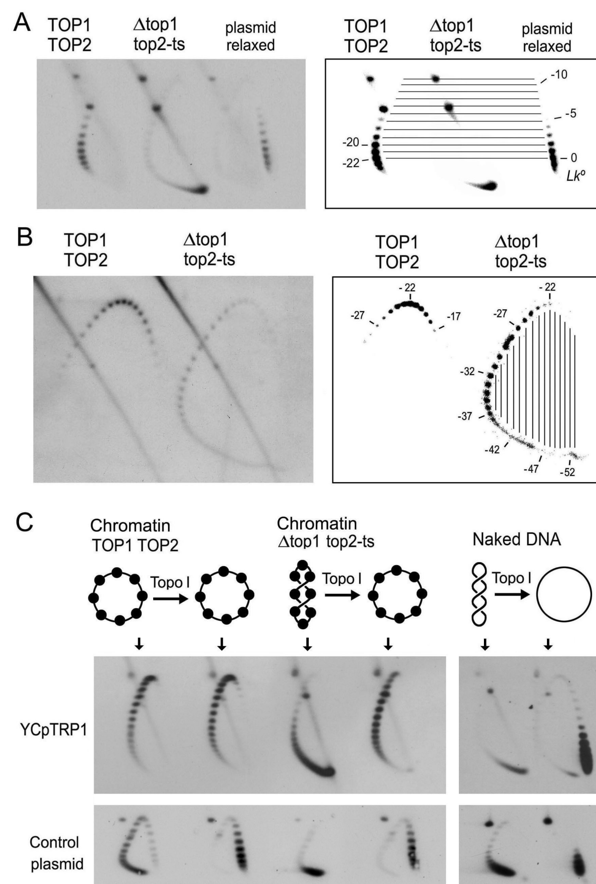

Figure 3. DNA supercoiling density and relaxation of yeast minichromosomes with reduced *Lk* values. A) The 2D gel-blot compares the *Lk* distributions of YCpTRP1 from *TOP1 TOP2* and Δ*top1 top2-4* yeast mutants after a 1-h incubation at 37ºC and of the naked YCpTRP1 plasmid relaxed with topo I at 37ºC. The diagram (rigth) was used to calculate Δ*Lk* between the relaxed plasmid (*Lk*^{o}) and the minichromosome in *TOP1 TOP2* cells. B) 2D-gel electrophoresis of the same yeast samples of YCpTRP1 described in A, but by increasing the chloroquine concentration to 3 µg/ml in the first dimension and to 15 µg/ml in the second. The diagram (rigth) was used to calculate Δ*Lk* between the minichromosomes in *TOP1 TOP2* cells and Δ*top1 top2-4* mutants. C) The 2D gel-blots show the *Lk* distributions of of YCpTRP1 minichromosomes (chromatin) that were solubilized from Δ*top1 top2-4* (1 h at 37ºC) and *TOP1 TOP2* yeast cells, and then incubated at 30ºC with and without vaccinia virus topo I for 30 min. The lower panel shows the topology of the control plasmid included in the incubations. For comparison, panels in the right shown the topology of YCpTRP1 (naked DNA) and the control plasmid that were separately relaxed with topo I. Electrophoresis was done with 0.8 µg/ml chloroquine in the first dimension and with 4 µg/ml in the second.

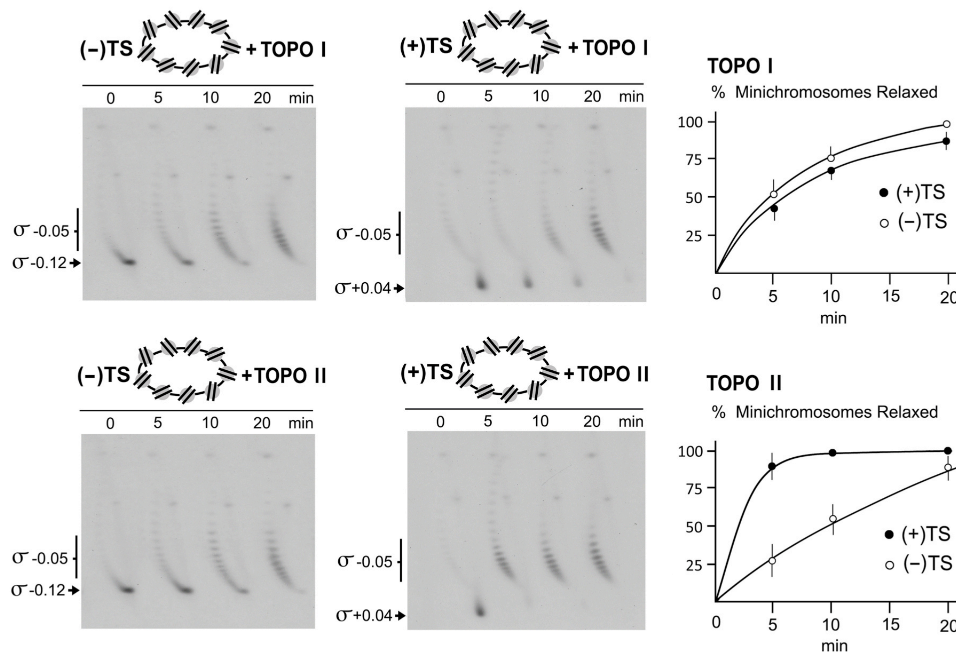

Figure 4. Relaxation of chromatin under (+) and (-) TS by topo I and topo II enzymes. YCpTRP1 minichromosomes under (-)TS (σ -0.05 to -0.12) or under (+)TS (σ > +0.04) were included (normalized amounts) in reaction mixtures (100 µl) containing Tris-HCl 10 mM, NaCl 150 mM, DTT 1mM, 8 mM $MgCl_2$, 1 mM ATP. The mixtures were pre-incubated at 30ºC for 5 min and DNA relaxation reactions were initiated by addition of catalytic amounts of purified yeast topo I or topo II enzymes. Reaction fractions (25µl) were quenched at different time points (min). The 2D gel-blots show the time course relaxation of YCpTRP1 minichromosomes under (-)TS and under (+)TS with topo I and topo II. The graphs show (mean and s.d. of three experiments) the time course relaxation of populations under (-)TS (σ -0.12) and under (+)TS (σ +0.04) produced by topo I and topo II. Relaxation of minichromosomes under (-)TS was quantified by measuring the intensity of the gel band (arrow) accumulated at the left-end of the *Lk* distribution relative to the entire *Lk* distribution signal. Relaxation of populations under (+)TS was quantified by measuring the intensity of the gel band (arrow) accumulated at the right-end of the *Lk* distribution relative to the entire *Lk* distribution signal.

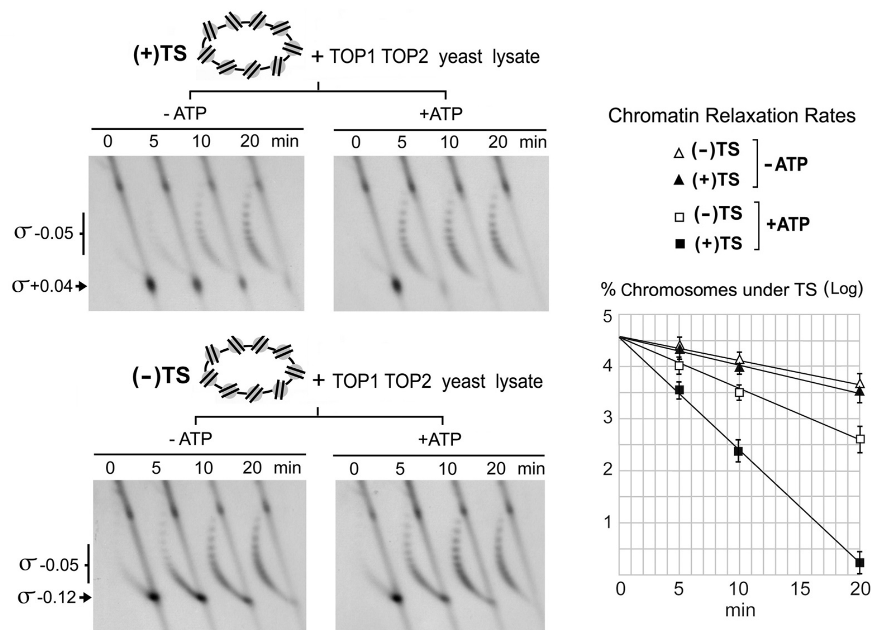

Figure 5. Relaxation rates of chromatin under (+) and (-) TS by endogenous topoisomerase activity. YCpTRP1 minichromosomes under (-)TS (σ -0.05 to -0.12) or under (+)TS (σ > +0.04) were pre-incubated at 30ºC for 5 min in reaction mixtures (100 µl) containing Tris-HCl 10 mM, NaCl 150 mM, DTT 1mM and 8 mM $MgCl_2$. Fresh lysates of *TOP1 TOP2* yeast cells were then added, and one half of each reaction mixture was supplemented with 1 mM ATP. Incubations continued at 30ºC and were stopped at the indicated time points (min). The 2D gel-blots show the time course relaxation of the minichromosomes under (-)TS and under (+)TS. The plot shows (mean and s.d. of three experiments) the time course reduction of populations under (-)TS and under (+)TS produced with yeast lysates in the presence and absence of ATP. Reduction of minichromosomes under (+) and (-)TS was quantified by measuring the gel-blot signals as described in figure 4.

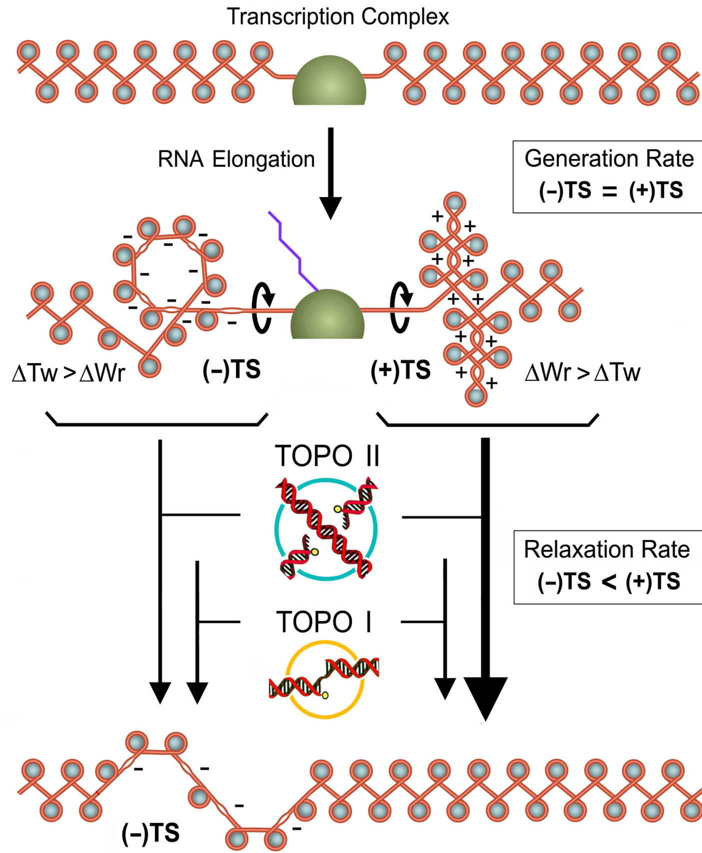

Figure 6. Role of chromatin conformation in the unbalanced relaxation (+) and (-) TS. During DNA transcription, (+) and (-)TS are generated at equal rates in front and behind the RNA elongating complex respectively. Plus (+) and minus (-) symbols reflect that the same number of positive and negative DNA extra helical turns are simultaneously produced. However, the conformational response of chromatin is distinct for (+) and (-)TS. Chromatin under (+)TS alters mainly the writhe of DNA ($\Delta Wr > \Delta Tw$) by extruding (+) supercoils and triggering nucleosome-reversome transitions. These deformations configure multiple DNA crossovers that are substrates for the DNA cross-inversion mechanism of topo II (blue). Conversely, chromatin under (-)TS alters mainly the twist of DNA ($\Delta Tw > \Delta Wr$) that leads toward double-helical unwinding. This deformation, along with a tight left-handed wrapping of DNA around histone octamers (grey), does not configure many DNA crossovers for topo II activity. Consequently, topo II relaxes (+)TS more quickly than (-)TS. In this scenario, the strand-rotation mechanism of topo I (yellow) slowly relaxes (-) and (+)TS at similar rates. As a result, the chromatin domain under (-)TS persists longer than that under (+)TS.