Topological constraints impair RNA polymerase II transcription and causes instability of plasmid-borne convergent genes

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ABSTRACT

Despite the theoretical bases for the association of topoisomerases and supercoiling changes with transcription and replication, our knowledge of the impact of topological constraints on transcription and replication is incomplete. Although mutation of topoisomerases affects expression and stability of the rDNA region it is not clear whether the same is the case for RNAPII transcription and genome integrity in other regions. We developed new assays in which two convergent RNAPII-driven genes are transcribed simultaneously. Plasmid-based systems were constructed with and without a transcription terminator between the two convergent transcription units, so that the impact of transcription interference could also be evaluated. Using these assays we show that Topos I and II play roles in RNAPII transcription in vivo and reduce the stability of RNAPII-transcribed genes in Saccharomyces cerevisiae. Supercoiling accumulation in convergent transcription units impairs RNAPII transcription in top1Δ strains, but Topo II is also required for efficient transcription independent of Topo I and of detectable supercoiling accumulation. Our work shows that topological constraints negatively affect RNAPII transcription and genetic integrity, and provides an assay to study gene regulation by transcription interference.

INTRODUCTION

DNA in its natural form in the cell is supercoiled. In eukaryotes this state facilitates its organization into chromatin around nucleosomes. DNA topoisomerases control supercoiling and their action is required in replication and transcription as well as in chromosome condensation and segregation (1,2). There are two types of DNA topoisomerases (Topo), I and II, which differ in their catalytic way of action, the first relying on a single-strand DNA cleavage, the second on a double-strand break (DSB). Topoisomerases I and II are the most representative of each type in all organisms. They are functionally interchangeable in eukaryotes, but not in bacteria.

During replication DNA topoisomerases can act at different stages. In Escherichia coli reconstituted replication systems require DNA supercoiling for replication initiation and the action of DNA topoisomerases (3). However, it is during elongation of replication that topoisomerases are expected to have a key role. They are required to remove the positive supercoiling that are generated by the continuous opening of the template strands. The intertwined daughter DNA molecules resulting after replication completion require the action of type II topoisomerases for their segregation. This late function in replication cannot be performed by type I topoisomerases but only by type II (4,5).

In contrast to replication, transcription does not involve a continuous separation of DNA strands. However, RNA polymerase progression through the DNA template would lead to the accumulation of local positive supercoiling ahead of the RNA polymerase and negative supercoiling behind. The twin-supercoiled-domain model was proposed to explain removal of this local supercoiling accumulation by topoisomerases during transcription (6). Evidence for transcriptional supercoiling associated with transcription has been provided in E. coli (6,7) and yeast (8,10). The relaxation of transcription-dependent supercoiling is generally considered to be the main function of Topo I, which is considered to play a swivel role in transcription (1,2). In eukaryotes such as the yeast Saccharomyces cerevisiae, the fact that top1A mutants are viable suggests that Topo II can replace the function of Topo I; whereas the opposite is not the case, since Topo I...
cannot replace Topo II at the end of replication during segregation.

Despite the theoretical bases for the association of topoisomerases and supercoiling changes with transcription, experimental evidence for the impact of topoisomerases deficiency and topological constraints in transcription is incomplete. In bacteria, the importance of Topo I has been well documented in rDNA transcription (11) or transcription from operons involved in amino acid metabolism (12,13). In yeast, an effect of topoisomerase activity has been reported on transcription initiation. This may be related to the need of negatively supercoiled DNA at promoter regions to facilitate transcription initiation (14). The effect of DNA topoisomerases on transcription elongation has been established for rDNA, in which the rate of RNAPI transcription is reduced when both DNA topoisomerases are inactivated in yeast (8,15). Instead, none or a poor effect has been reported for mRNA transcription as tested in endogenous and lacZ reporters fused to GAL promoters in vivo (10,16).

In addition to the effect of Topo mutations in cell growth, transcription and replication, they have an important impact on genome integrity. Stability of tandem rDNA repeats in yeast has been shown to be strongly dependent on DNA topoisomases I and II, whereas this was not the case for direct repeats outside of the rDNA region (17). Hyper-recombination has been shown in plasmid-borne direct-repeats in double top1 top2 mutants (18). It has been proposed that this is mainly the result of the failure to relax the negative rather than the positive supercoiling of DNA (18). Nevertheless, impairment of replication fork progression by obstacles or DNA lesions can lead to replication fork collapse that in turn could trigger recombination as a mechanism of replication restart (19). Thus, hyper-recombination of yeast topoisomerase mutants could be linked to their role in replication, but the functional relevance of Topo I in rDNA transcription and its large accumulation in the nucleolus, makes plausible that hyper-recombination could also be linked to transcription. Indeed, rDNA genes contain the RNAPI transcription-dependent HOT1 recombination hotspots (20,21), and one key function of the Fob1 barrier to DNA replication is to avoid replication–transcription collisions that could potentially compromise genome integrity (22).

In this sense, one intriguing class of hyper-recombination mutations in yeast is composed of those of the THO complex. THO is a conserved protein complex composed of stoichiometric amounts of Hpr1, Tho2, Mft1 and Thp2, with a role in transcription elongation, mRNPs biogenesis and RNA export (23). THO mutants, as well as mutants related to mRNPs biogenesis factors such as the THSC/TREX-2 complex, cause a strong hyper-recombination between direct repeats that are transcription-dependent. This phenotype is linked to the co-transcriptional formation of R-loops that can impair the progression of replication (24,25). Interestingly, in E. coli mutants lacking topoisomerase I, an excess of negative supercoiling during transcription can lead to R-loops (26). Furthermore, THO and THSC mutants show synthetic growth defects in combination with top1 and top2 mutations (27). All this evidence raises the question of whether hyper-recombination in the rDNA region in topoisomerase mutants could be linked to transcription.

Thus, although topological constraints and topoisomerase mutations have a clear impact in expression and stability of the rDNA locus this is not that clear for RNAPII transcription and genome integrity at regions others than rDNA. To investigate this, new and highly sensitive in vivo assays were developed based on plasmid-borne DNA sequences of increasing lengths that are transcribed form strong promoters with or without convergent transcription coming from a second promoter. We show that both top1 and top2 mutations drastically reduce RNAPII transcription, a defect that is strengthened as the length of the transcribed DNA sequence increases and by convergent transcription. Importantly, top2-1 mutation causes a significant increase in direct-repeat recombination in a top1A mutant background; however, this hyper-recombination was predominantly independent of transcription of the direct-repeat system itself. Therefore, this study provides new evidence for the in vivo relevance of Topos I and II in RNAPII transcription and genome integrity. We propose that DNA topoisomerases has a non-interchangeable role in the maintenance of integrity of RNAPII genes providing evidence for the negative effect of topological constraints in transcription and genome stability in DNA regions beyond the rDNA. Although a similar study would need to be performed in chromosome-borne constructs, we believe that these results may also be valid for chromosomal regions, provided that local supercoiling dynamics follow universal rules.

MATERIALS AND METHODS

Strains and plasmids

Yeast strains and plasmids are described in Supplementary Tables S1 and S2, respectively.

Chromatin immunoprecipitation analysis

For ChIP experiments, strains were grown in synthetic medium (SC) 2% glycerol-2% lactate to an OD660 of 0.5 (for GALI). For GALI ChIPs the culture was split in two, and one half was supplemented with 2% glucose (repressed transcription) and the other with 2% galactose (activated transcription). For Ptet-based systems, yeast strains were grown in synthetic medium (SC), the culture was split in two, and one half was supplemented with doxycycline. Samples were then taken after 4h of induction and ChIP assays were performed as described (28). Polyclonal anti-PolII (N-20) (Santa Cruz Biotechnology) and Protein A–Sepharose were used for RNAPII immunoprecipitation. The GFX purification system (Amersham) was used for the last DNA purification step. We used the PCR of the intergenic region at positions 9716–9863 of chromosome V as a negative control. Real-time quantitative PCR and calculations of the relative abundance of each DNA fragment was performed as described (29). For each experiment, the DNA ratios in the different regions were calculated from the DNA amount of these regions relative to the intergenic region.
Median and Standard Deviation (SD) of three independent experiments are shown.

Nucleic acid isolation

For DNA isolation, pellets were re-suspended to a final concentration of 1.5–2 × 10⁹/ml in NIB pH 7.2 (17% glycerol, 50 mM MOPS, 150 mM KAc, 2 mM MgCl₂, 0.5 mM spermidine and 0.15 mM spermine) with 3 mg/ml Zymoliase-20T and incubated at room temperature for 25 min. Cellular suspensions were diluted seven times with chilled water and centrifuged to collect nuclei. Pellets were re-suspended in 720 μl of TE (50 mM Tris–HCl, pH 8, 20 mM EDTA) and mixed with 80 μl of 10% SDS. After phenol–chloroform–isoamylalcohol (25:24:1) extraction and ethanol precipitation, DNA was finally re-suspend in 20–30 μl of TE.

Chloroquine gel electrophoresis

Electrophoresis was performed at room temperature on horizontal slab gels (0.7% agarose) containing TPE buffer (50 mM Tris–phosphate [pH 7.2], 1 mM EDTA and 25 mM phosphoric acid). Chloroquine (Sigma) was added to the melted agarose from a 10 mg/ml stock prior to casting the gel and was present in the electrophoresis buffer. The buffer was re-circulated continuously during electrophoresis. The concentration of chloroquine was 4μg/ml. Each lane had 20μg of total nucleic acids. The gels were run at 40 V for 48 h.

Miscellaneous

Yeast methods, α-32P-labeled DNA probes, northern and Southern analyses were performed according to standard procedures. RNA was isolated from mid-log phase cells.

Recombination frequencies were obtained by fluctuation tests as the median value of six independent colonies isolated from SC plates. The final frequency given for each strain and condition is the mean and SD of three to four median values as described (30). Recombinants Leu+ were selected in SC- Leu plates.

RESULTS

Convergent transcription in systems with or without terminator sequences

For a fine assessment of the possible effect of topological constraints in transcription, a plasmid-based system was constructed in which a 4-kb sequence was transcribed from two convergent and differentially regulated promoters located on each side. For this construction, we fused a 2-kb lacZ fragment under the GALI promoter to a 2-kb lys2 fragment under the quimeric tet promoter so that transcription driven from the two promoters was convergent (Figure 1). Two versions of this system were constructed in monocyte CEN-based plasmids: one with a 0.4-kb CYC1 terminator between the lys2 and lacZ sequences (pTGlylaTER) and the other without transcription terminator (pTGlyla). The CYC1 terminator sequence contains two converging terminators so that it acts in both orientations. As expected, whereas in the pTGlyla system without terminator the 4-kb lys2-lacZ mRNA was produced when cells were cultivated either with 2% galactose (GALI promoter activated) or without doxycycline (tet promoter activated), but not in 2% glucose + doxycycline (GALI and tet promoters repressed) (Figure 1A). To confirm that the band detected in 2% galactose was driven from the GALI promoter and the band detected in the absence of doxycycline was driven from the tet promoter northern analysis was performed in which hybridization was made with specific ssDNA probes. As expected, the 4-kb mRNA band was specific to a DNA strand. It was detected by the Watson-chain-specific (W) probe in the absence of doxycycline (Ptet active), and with the Crick-chain-specific (C) probe in galactose (PGALI active), according to the promoter from which each transcript was transcribed (Figure 1B). Instead, in the pTGlylaTER system either the 2-kb lacZ or the 2-kb lys2 mRNAs were detected in galactose regardless of the presence of doxycycline, or in doxycycline regardless of the carbon source, respectively (Figure 1C and D).

Importantly, in the pTGlyla system, the level of 4-kb lys2-lacZ transcripts was lower when both the PGALI and Ptet promoters were simultaneously active than when only one was active, as observed with either the lys2 or the lacZ probe (Figure 1A and B). This result is consistent with transcription interference either at the promoter or transcriptional stalling provided by RNAPII collisions. Since the 4-kb transcript driven from either the tet or GALI promoters reaches the downstream converging promoter, the latter may undergo transcription interference. When the transcripts were analyzed with specific ssDNA probes it was found that after galactose activation the Ptet-driven 4-kb transcript, as detected with the W probe, was reduced, whereas the PGALI-driven transcript was below detection levels (Figure 1B). This indicates that since the Ptet-driven RNAPII reaches PGALI, it causes promoter interference impeding PGALI activation, even though transcriptional stalling cannot be excluded either. Similarly, PGALI-driven transcription seems to reduce the efficiency of Ptet-driven transcription, although to a lesser extent.

To assay whether the northern results could be interpreted in terms of RNAPII progression, a chromatin–immunoprecipitation (ChIP) analysis was performed with anti-Rpb1 N-ter antibodies in both the pTGlylaTER and pTGlyla systems under all possible conditions (with either none, only one or both promoters activated). As can be seen in Figure 2, RNAPII recruitment was not observed in either of the regions analyzed when both promoters were repressed in either system, as expected. In the pTGlylaTER system RNAPII was detected in lys2 when only Ptet was active and in both lys2 and lacZ when only PGALI was active (Figure 2A). The latter was consistent with transcription termination read-through observed for transcription initiated at the strong PGALI promoter, as observed by northern (Figure 1). In the pTGlyla system, RNAPII recruitment was observed at both the lys2 and lacZ regions when either Ptet or PGALI were the only active promoters, whereas recruitment to one promoter was only detected when transcription was driven from the opposite promoter (Figure 2B), implying that such polymerases corresponded to elongating RNAPIIs. When both promoters
were active, the lys2 and lacZ signals were detected, as expected, in the pTGlylaTER system, whereas only the lys2 signal was detected in pTGlyla. This confirms that the northern analysis used can be interpreted in terms of RNAPII capacity to transcribe the different regions analyzed.

Transcription elongation is sensitive to Topos I and II inactivation

Next, the two constructs were used to analyze transcription in the presence of doxycycline, so that the Ptet promoter was inactivated and transcription was only driven from P_GAL1. Under these conditions kinetic experiments were performed of P_GAL1-driven transcription with 2% galactose to determine the efficiency of transcription of the 4-kb lacZ-lys2 transcript in pTGlyla and the 2-kb LacZ transcript in pTGlylaTER. As can be seen in Figure 3, transcription of LacZ was clearly reduced in top1Δ mutants, more reduced in top2-1 mutants and further in the double top1Δ top2-1 mutants. Transcription of the 4-kb lys2-lacZ was dramatically decreased in all single and double top mutants. This result is consistent with a defect in transcription elongation so that the longer the RNA is transcribed the stronger the effect. The double mutant showed the strongest effect and a similar dramatic decrease in the accumulation of both of the 2- and 4-kb transcripts.

Different studies have revealed that yeast mutations impairing transcription elongation causes a reduction in mRNA accumulation that in most cases increases with the length of the transcript unit analyzed (23). Using plasmid-borne P_GAL1::PHO5 and P_GAL1::lacZ constructs containing the 1.2-kb and 3.1-kb yeast PHO5 and bacterial lacZ ORFs, respectively, under the same GAL1 promoter, the negative effect of topoisomerase mutations was confirmed in transcription of long DNA sequences. The single and double Topo mutants have a significant effect on the efficiency of transcription of the PHO5 ORF, the reduction in the accumulation of the lacZ mRNA being stronger in the single top2-1 and in the double thplA top2-1 (Supplementary Figure S1). Given that transcription in both constructs is driven from the same GAL1 promoter, this result excludes the possibility

Figure 1. Transcription analyses of the lys2-CYC-lacZ and lys2-lacZ fusion constructs. (A) Northern analyses of lys2-lacZ-containing mRNAs driven from the tet and GAL1 promoters. The 3-kb XbaI-EcoRV lacZ and a 589-bp 25 S rDNA internal fragments obtained by PCR (rRNA) were used as DNA probes. (B) Northern analyses of lys2-lacZ. The oligos C and W were used as single-stranded DNA probes. (C), (D) Northern analyses of pTGlylaT-1 and pTGlylaT-5 plasmids. Arrows refers to the mRNA being produced from the specified promoter. No arrow indicates that there is no transcription from that promoter. All experiments are made in the absence of doxycycline, so that the tet promoter is permanently active. A total of 2% glucose (Glu) or galactose (Gal) are used to either repress or activate the GAL1 promoter. Other details as described in ‘Materials and Methods’ section.
that the major transcription defects observed in top mutants occur at transcription initiation, but at elongation. In addition, consistent with the idea that eukaryotic Topos I and II can partially replace each other, we showed that overexpression of Top1 can partially suppress the lacZ mRNA accumulation defect of top2-1 mutants (Supplementary Figure S2).

Convergent transcription is impaired under Topos I and II inactivation as a function of supercoiling accumulation

Next, we assayed whether or not convergent transcription affected transcription efficiency. This analysis of convergent transcription was made in the absence of doxycycline to allow continuous Ptet-driven transcription. Time-course kinetics was performed for the PGAL1-driven transcripts after galactose addition. As can be seen in Figure 4, in the pTGlylaTER system with the transcription terminator the overall levels of the PGAL1-driven lys2 transcript did not show a significant change after activation of the converging PGAL1. At 200 min of PGAL1 activation the overall levels of lys2 transcripts were similar to the levels before activation. At intermediate times some differences between wild-type and mutant strains were evident, but these were minor. This may be a response to cell adaptation to new conditions. Importantly, at 200 min of activation the lys2 transcript levels were the same in wild-type and mutant strains, with a slight decrease in top1A top2-1 mutants (10% of wild-type levels) (Figure 4A). The clear impairment in transcription of the 2-kb lacZ transcript caused by topoisomerase mutations as compared to the poor or no effect on the 2-kb lys2 transcript (both transcripts are of the same size but are driven from promoters of different strengths), indicates that the higher the transcription rate the higher the requirement of topoisomerases.

In the pTGlyla system there is little change in the total amount of 4-kb transcripts (Figure 4B) during the kinetics performed. As shown above, these results indicate that pTGlyla, which contains no terminator sequence between the converging promoters, undergoes promoter interference caused at PGAL1 by the RNAPII coming from Ptet. However, despite the interference phenomenon, transcripts were moderately low in single top1 and top2 mutants (60–65% of wild-type levels after 200 min activation) and lower in double top1 top2 mutants (38–45%) as compared to wild-type levels. This is consistent with the importance of topoisomerase activities in converging transcription. It is worth noting the different topoisomerase requirements of transcription of the 4-kb lys2-lacZ and 2-kb lys2 fragments in the pTGlyla and pTGlylaTER systems (compare Figures 4A. up and D, bottom). This is due to the longer length of the lacZ-lys2 fragment, consistent with the conclusion that the longer the transcript the higher the dependency of transcription on topoisomerase activity (Figure 1). Altogether, the results indicate that topoisomerases I and II are required for efficient RNAPII transcription elongation.
supercoiling, rather than to a specific function of any of the topoisomerases at specific target DNA sites, supercoiling of the plasmids was analyzed carrying both the pTGlylaTER and pTGlyla systems under conditions of transcription activation of both promoters. As can be seen in Figure 5, a large proportion of supercoiled DNA is accumulated in top mutants, in particular in top1 and top1 top2 when both promoters are active, but not when they were inactive. Experiments were performed similarly to previous reports using chloroquine concentrations in which all topoisomers show negative supercoiling (9). Importantly, accumulation of the supercoiled plasmid band is clearly stronger in pTGlyla versus pTGlylaTER, consistent with the conclusion that the longer the length of the transcription unit, the higher the change in supercoiling produced by transcription and the stronger the requirement of topoisomerases. Even though on a theoretical basis the impact of transcription on DNA supercoiling is local, TOP1 inactivation has a global impact on plasmid supercoiling as has been previously reported (9). As previously shown (9) accumulation of global supercoiling was not evident in top2-1 mutants.

Efficient RNAPII-driven convergent transcription requires Topos I and II

So far, all our previous experiments were performed at 30°C, a semi-permissive temperature to allow growth of...
Figure 4. Northern analyses of convergent transcription in the lys2-CYCt-lacZ and lys2-lacZ fusion constructs. (A) Northern analyses of lys2-CYCt-lacZ mRNAs driven from the tet and GAL1 promoters in wild-type (HRN1-4A), top1D (TOHR-11A), top2-1 (TORH-6A) and top1D top2-1 (TOHR-13C) strains. The 3-Kb XbaI-EcoRV lacZ internal fragment were used as DNA probe. (B) Northern analyses of lys2-lacZ mRNAs driven from the Ptet and PGAL1 promoters. Other details as described for Figure 3.
thermosensitive mutants. Yet, it was possible to see a synergistic effect on double top1 top2 mutants suggesting that Topo II has a role in transcription in vivo. This conclusion was confirmed by assaying the effect on complete Top II inactivation. These experiments were repeated on convergent transcription in both the pTGlyla and pTGlylaTER systems after shifting cells to the restrictive temperature of 37°C. As can be seen in Figure 6A, in the pTGlylaTER system in which both convergent promoters are activated, whereas lys2 and lacZ transcript levels show little effect on top1D and top2-1 single mutants, which were dramatically reduced in the double top1D top2-1 mutants (20% of wild-type levels after 2 h at 37°C). In the pTGlyla system, whereas in wild-type and, to a lesser extent, in the top1D mutant, the 4-kb transcript levels increased with time at 37°C consistent with a faster metabolism at this temperature, they did not change in top2-1 mutants and dropped dramatically in top1D top2-1 mutants (Figure 6B). These results were confirmed by analyzing the effect of top2-1 at different temperatures, from 30°C to 33°C in which it could be seen a dramatic decrease in the accumulation of lacZ mRNA in the top1A background as the temperature increased (Supplementary Figure S2). Altogether, the results indicate that Topo II plays a critical role in RNAPII transcription in vivo, which indeed is quantitatively more important than that of Topo I.

top2-1 and top1D confer a general hyper-recombination phenotype

It is known that top1D mutants confer a strong hyper-recombination at the rDNA locus (17). To test whether this lack of effect was general for any DNA region and for different types of recombination events and whether it was related to transcription, the effect of top mutations on recombination was analyzed in different and highly sensitive plasmid-borne recombination systems. First we assayed recombination in the pTINV system based on two inverted repeats of the LEU2 gene, one copy of which is a 5'-end truncation (leu2D5') and the other a 21-bp insertion mutation under the control of the regulated tet promoter (Ptet::leu2-HOr) (31). This system allows detection of gene conversion events of the leu2-HOr allele with or without crossovers as a function of transcription, which leads to Leu+ recombinants that are scored on SC-leu plates (Figure 7A). As can be seen in Figure 7A, whereas Leu+ recombination levels in top1D and top2-1 mutants were similar to the wild-type both with or without transcription, recombination was increased 26- and 37-fold without or with transcription. Next, we tested the effect on recombination in the pGL-PHO5 system carrying two 0.6-kb leu2 direct repeats under the control of the GAL1 promoter and the PHO5 ORF located between the repeats (32). This system is used to study deletions occurring by recombination between the two leu2
Figure 6. Effect of top1 and top2 mutations on transcription of lys-CYCt-lacZ and lys-lacZ fusion constructs at 37°C. (A) Northern analyses of lys-CYCt-lacZ-containing mRNAs driven from the tet and GAL1 promoters in wild-type (HRN1-4A), top1Δ (TOHR-11A), top2-1 (TORH-6A) and top1Δ top2-1 (TOHR-13C) strains at 37°C. Mid-log phase plasmid-transformed cells were diluted to an OD_{600} of 0.4 synthetic complete (SGal)-Trp medium at 26°C and shifted to 37°C, samples were taken for northern analyses at different times. (B) Northern analyses of lys-lacZ mRNAs driven from the P_{tet} and P_{GAL1} promoters. Other details as described in Figure 3.
direct repeats that are scored as Leu+ colonies (Figure 7B). It can be used either with active transcription of the direct-repeat construct in media supplemented with 2% galactose or without transcription in media with 2% glucose. Leu+ deletions occurred at similar levels in top1A and top2-1 mutants as in the wild-type, whereas in top1A top2-1 mutants there was a significant increase in recombination both with or without transcription (15- and 10-fold increase, respectively) (Figure 7B). These data indicate that only a simultaneous defect of topoisomerases I and II lead to a general increase in recombination, which is primarily independent of transcription of the DNA sequences involved in the recombination event. Similar results were observed with the pGL-lacZ system (32), the same as pGL-PHO5 with LacZ between the leu2 repeats (Figure 7C). A clear increase in recombination was observed in top1 top2 mutants with or without transcription (7.2- and 11-fold increase). However, it is worth noting that a significant increase (4.2-fold) was also observed in single top1 mutants but only under transcription conditions, suggesting that the torsional stress caused by transcription elongation through the lacZ sequence is recombinogenic in the absence of Topo I.

To test whether hyper-recombination could also be observed in direct-repeat systems containing the TGlyla and TGlylaTER converging transcription systems, new plasmid-borne recombination systems were constructed by inserting the TGlyla and TGlylaTER constructs between the two 0.6-kb leu2 direct-repeats of the plasmid-borne L repeat construct previously published (33) to create the recombination systems L-lyla and L-lylaTER (Figure 8). The frequency of Leu+ deletions was determined under conditions in which none (media with glucose and doxycycline), only one (with galactose and doxycycline or glucose without doxycycline) or both (with galactose without doxycycline) promoters were activated. As can be seen in Figure 8A, a 3.4- to 5.6-fold increase in Leu+ deletions was observed in the double top1D top2-1 mutants under the four different conditions of transcription tested as well as in the single top1D mutant when GAL1p-driven transcription was active, regardless of transcription from the tet promoter. Similar results were observed in the L-lylaTER (Figure 8B). In these cases, a 3.3- to 12-fold increase in recombination was observed in the double top1A top2-1 mutants under the four different conditions of transcription tested as well as in the single top1A mutant when GAL1p-driven transcription was active, regardless of transcription from the tet promoter. Altogether, these results indicate that only a simultaneous defect of topoisomerases I and II lead to a general increase in recombination, and

Figure 7. Recombination analyses of wild-type, top1A, top2-1 and top1A top2-1 strains. (A) Recombination between inverted repeat in plasmid pRS316-TINV carrying two inverted leu2 sequences. One copy is the leu2-HOr allele under the tet promoter and the other is a 5'-end truncated allele (leu2Δ5'). Transcription of leu2 is driven from tet promoter.

Figure 7. Continued
Leu+ recombinants can arise by gene conversion of leu2-HOr without an associated inversion or by crossover occurring upstream of the HO site, whether or not associated with gene conversion. (B) Recombination between direct repeats in the system GL::PHO5 carrying a 600-bp truncated leu2 repeat under the PGAL1 promoter. Leu+ recombinants can arise by the deletion of the PHO5 sequence. (C) Recombination between direct repeats in the system GL::LacZ carrying 600-bp truncated leu2 repeats under the PGAL1 promoter. Leu+ recombinants arise by the deletion of the LacZ sequence.
this effect is primarily independent of transcription of the DNA repeats and their intervening DNA sequences involved in the recombination event.

In this sense, mutations in the THO complex, such as hpr1Δ, cause a strong increase in direct-repeat recombination that is linked to transcription. As Topo mutations lead to weaker hyper-recombination that is not restricted to direct-repeat systems and mainly in a transcription-independent manner, it seemed reasonable that both type of mutations may affect recombination via different pathways. To test this possibility recombination in the L-lyla system was analyzed in double mutant combinations of hpr1Δ with top1Δ and top2-1 mutations. As can be seen in Supplementary Figure S3, hyper-recombination was clearly observed in hpr1Δ mutants under Ptet or PGAL1-driven transcription (12- to 15-fold), but to a lesser extent when both the tet and GAL1 promoters were inactive. The triple mutant hpr1Δ top1Δ top2-1 shows a synergistic effect on hyper-recombination that reached 47- to 71-fold increase when only one promoter was active, and 123-fold when both promoters were active. Although we cannot exclude that transcription outside of the DNA recombination construct in the plasmid could influence the general levels of recombination due to its global impact on plasmid supercoiling, our result shows that, in contrast to THO-complex mutations, the effect of Topos I and II mutations in non-rDNA recombination is primarily not linked to transcription of the recombination system.

**DISCUSSION**

Here we show that Topos I and II play a role in eukaryotic RNAPII-driven transcription and genome stability in the yeast *S. cerevisiae*. RNAPII transcription requires Topos I and II at the elongation step, this being the role of Topo II in elongation independent of Topo I and of supercoiling accumulation. Notably, the impact of supercoiling accumulation in genome instability is not related to transcription of the direct-repeat in which instability is measured. Consequently, our work reveals independent effects of the overlapping functions of Topos I and II in transcription and genome integrity. In addition, we show that a promoter cannot be efficiently activated if the anti-sense DNA strand is transcribed, a result that suggests new possibilities to explain regulation of gene expression by promoter interference.

RNAPII transcription elongation impairment by topological constraints *in vivo*

The relevance of topoisomerases and supercoiling constraint in transcription was anticipated and demonstrated...
in the past in prokaryotic and eukaryotic cells (2,5). Different reports have shown that the levels of DNA supercoiling have an impact in RNA synthesis in E. coli (34,35,36). Requirement of DNA topoisomerases in transcription can be explained by the twin-supercoiled-domain model (6). The negative impact of failure to control DNA supercoiling in transcription is believed to be linked to the transcription elongation step. Evidence for this has been provided in the rDNA regions, as in yeast (2,14). Other studies of transcription at the rDNA region in human cells are consistent with this view (37). In yeast, it has also been shown that positive supercoiling diminishes mRNA transcription, probably at the initiation step (15). In human cells it has been shown that RNA polymerase I is involved in both repression and activation of transcription (38), providing evidence that DNA supercoiling can also control transcription initiation. All these data plus the observation that yeast topoisomerase I is involved in both repression and activation of transcription (38) indicates that this supercoiling needs to be removed by the action of DNA topoisomerases to allow transcription. Whereas the transcriptional role of Topo I has been previously documented, no evidence has been reported for a role of Topo II in RNAPII transcription in vivo, despite the fact that Topos I and II are redundant in eukaryotes. Evidence for a role of Topo II in RNAPII transcription has been shown in chromatin templates in vitro (39). In addition, despite the expectation that transcription elongation should be sensitive to supercoiling, according to the twin-supercoiled-domain model, experimental evidence for the negative effect that supercoiling may have in RNAPII-driven transcription elongation was lacking.

Our study on RNAPII transcription has been based on two convergent transcription units. Convergent transcription should create a local accumulation of positive supercoiling ahead of each polymerase that would quickly accumulate in topoisomerases mutants. Our work shows that, indeed, convergent transcription is reduced when both promoters are active in both single top1 and top2 mutants. Such a reduction in transcription efficiency is synergistic in double mutants top1 top2, in which transcription is reduced to almost undetectable levels. The results reveal a role for Topo II in transcription. It is likely that Topo II function has a minor impact in wild-type cells expressing Topo I. Nevertheless, our result suggests that Topo2 can replace Topo I in transcription when the latter is absent in the cell. This negative effect of Topo mutations on transcription is seen when the length of the mRNA is 4 or 2 kb, which likely indicates that 2-kb of transcription run is already sufficient to see the effect of supercoiling accumulation in transcription. Interestingly, transcription in simple systems in which transcription is only unidirectional, no effect of topoisomerases mutations is observed for 1.2-kb transcript units, but only in 3-kb long transcription units, even though this effect is clearly lower than for convergent transcription. This is consistent with a major impact of convergent transcription on local DNA supercoiling and with the idea that the longer the DNA sequence transcribed the higher the supercoiling. The impact in supercoiling has been shown indeed by chloroquine gels, as expected from previous work (9) (Figure 5). Although we cannot exclude the possibility that the reduction in transcription efficiency could be determined at the level of transcription initiation, altogether the data indicate that transcription elongation is a major step impaired by supercoiling accumulation. Accordingly with, we have been able to observe a synergistic effect on transcription efficiency in a top1 top2 mutant carrying a deletion of the SPT4 elongation factor gene in the 1.2-kb transcript. This indicates that under impaired transcription elongation, supercoiling accumulation has a negative impact on transcription elongation (Supplementary Figure S4).

Therefore, our work shows that mRNA transcription is strongly impaired by topoisomerase inactivation and supercoiling accumulation. Both Top2 and Top1 play important roles in removing superhelicity during transcription. Therefore, Top2 not only substitutes for Top1 to control supercoiling accumulation during transcription elongation, but it also plays a function in wild-type cells, consistent with its reported roles in RNAPII transcription in chromatin templates (39). Interestingly it has recently been shown that Topo2 is required to relax nucleosomal DNA (40) and for rDNA transcription in yeast (2). Further investigation would be necessary to define how Top2 acts during transcription, and whether its role is only related to supercoiling accumulation, given the fact that under the conditions studied no strong accumulation of DNA supercoiling is observed in top2 mutants in contrast to top1 mutants (Figure 5).

It is worth noting that our assays provide an example in which promoter interference has a negative impact on the ability of a promoter to be activated. Thus, when the GAL1 promoter was active, in which case the anti-sense DNA strand of the tet promoter was transcribed, the tet promoter could not be efficiently activated. This suggests that promoter transcription interferes with its activity, opening the possibility that promoter inactivation by CUTs or anti-sense RNAs (41,42) might also be caused by transcription, which could presumably disrupt the proteins and architecture of a functional promoter.

**Direct-repeat hyper-recombination in topoisomerase-defective cells**

Previous evidence has shown that rDNA stability was highly dependent on DNA topoisomerases I and, to a lesser extent, on Topo II (17). Short circular plasmids carrying non-rDNA regions have been shown to suffer frequent multimerization in yeast Topo mutants (43). Although multimerization is attributed to recombination, no study has been performed on whether such multimerization could be due to replication impairment leading to re-initiation and/or sigma-like type of replication due to enhanced superhelicity. The fact that this is observed in short circles indicate a specific structural demand, presumably associated with changes in supercoiling that may not be related only to recombination. The increased in recombination observed in our systems is in any case lower and not accompanied by multimerization.
A different study using direct-repeat systems such as those used in this study, revealed increases in direct-repeat recombination of 40- to 100-fold only in top1 top2 mutants (18). An important difference between those direct-repeats did not contain DNA sequences between the tandem direct repeats whereas the systems used in this study contained between 2 and 4 kb. This may explain why in our case at most we get a 10-fold increase in recombination. We previously showed that the distance between the repeats has an impact on recombination: the longer the intervening sequence the higher the probability that recombination events are initiated there (33). Long repeats may have a steric impediment that would interfere with a proper pairing of the two repeats along their length, whereas such steric limitations may not exist when the distance between the repeats is longer than the repeat units. Failure to relax negative supercoiling might limit pairing of both DNA repeats. Interestingly, however, our study reveals that topoisomerase depletion strongly increases gene conversion (37-fold). As it seems plausible that gene conversion is mainly initiated by DSBs, our study suggests therefore a general impact of negative supercoiling on genome fragility linked to DNA breaks.

Given the impact of Topo depletion on transcription in general and transcription of the convergent constructs used in this study in particular, it is notable that the increase in recombination is not linked to the increase in the levels of transcription of the direct repeat itself. Even when convergent transcription is activated between the two direct repeats, with or without a transcription terminator in between, hyper-recombination is not significantly affected. This implies that the hyper-recombination caused by topoisomerase inactivation is not caused by breaks that might occur as a consequence of local conflicts generated between transcription and replication. This may contrast with the hyper-recombination in the rDNA (8).

In non-rDNA regions, evidence has recently shown that in human cells Top1 prevents replication obstacles in transcribed sequences, suggesting that such obstacles are mediated by R-loops (44). Indeed it has recently been shown in yeast that R-loops form in yeast cells deleted for TOP1 (45), consistent with previous work in E. coli showing R-loop accumulation in the rDNA in topA mutants (26). Even though it seems clear that transcribed regions are more prone to obstacles, and recombination in our assays are determined in transcribed genes, our data clearly shows that the topological constrains generated in topoisomerases mutants causes instability in DNA regions that are not transcribed either. In our systems in particular, hyper-recombination is observed in the direct repeat regardless of whether or not it is transcribed. Another report in yeast (46) showed that Top2 prevents chromosome fragility in regions that are transcribed during S phase. Again, we are able to observe hyper-recombination even in the absence of transcription. Therefore, there are two possibilities, either the chromosome fragility reported in human and yeast in top1 or top2 cells caused by replication impairment in transcribed DNA regions is not resolved primarily by detectable homologous recombination or if they are, transcription-dependent recombination only reflects a minor fraction of chromosome fragility generated by topoisomerase inactivation. The strong impact of topoisomerase inactivation on replication progression would therefore be sufficient to generate the hyper-recombination phenotype observed in top1 top2 mutants. This would fit the expected role of topoisomerases in regulating DNA supercoiling changes occurring during replication fork progression (4,5). Failure to resolve this supercoiling accumulation may cause replication stalls that can resolve in ssDNA breaks or gaps.

Our results exclude the possibility that a co-transcriptional R-loop that could putatively form at the direct repeats could be responsible for recombinogenic breaks. Global supercoiling of the plasmid may also be affected by transcription of DNA regions away from the direct repeat used for the analysis of recombination, but whether such a transcriptional activity influences at distance the levels of recombination of the direct repeat we cannot be certain. However, given the low levels of transcription of the plasmid marker as compared to the high transcript levels of the direct-repeat recombination system driven from the GAL1 promoter, this is an unlikely possibility. In any case recombination would not initiate at the transcribed plasmid marker but away of it in the non-transcribed direct repeat. For the same reason, an R-loop putatively formed in the plasmid outside the direct-repeat recombination system would not be expected to induce the high levels of recombination determined in this study. In conclusion, the genome instability phenotype of Topo mutants does not require transcription of the sequence involved in the recombination event.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1–4.

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