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Topoisomerase II is required for the production of long Pol II gene transcripts in yeast

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ABSTRACT

The extent to which the DNA relaxation activities of eukaryotic topoisomerases (topo I and topo II) are redundant during gene transcription is unclear. Although both enzymes can often substitute for each other *in vivo*, studies *in vitro* had revealed that the DNA cross-inversion mechanism of topo II relaxes chromatin more efficiently than the DNA strand-rotation mechanism of topo I. Here, we show that the inactivation of topo II in budding yeast produces an abrupt decrease of virtually all polyA+ RNA transcripts of length above ~3 kb, irrespective of their function. This reduction is not related to transcription initiation but to the stall of RNA polymerase II (Pol II) during elongation. This reduction does not occur in topo I mutants; and it is not avoided by overproducing yeast topo I or bacterial topo I, which relaxes (−) DNA supercoils. It is rescued by catalytically active topo II or a GyrBA enzyme, which relaxes (+) DNA supercoils. These findings demonstrate that DNA relaxation activities of topo I and topo II are not interchangeable *in vivo*. Apparently, only topo II relaxes efficiently the (+) DNA supercoils that stall the advancement of Pol II in long genes. A mechanistic model is proposed.

INTRODUCTION

During transcriptional elongation, the DNA double helix rotates axially relative to the RNA polymerase. At the chromosomal level, this rotation is hindered by the viscous drag of chromatin fibers and by interactions that anchor DNA and the transcription ensembles to subnuclear structures (1). Consequently, (+) torsional stress overwinds and positively supercoils the DNA ahead of the transcribing RNA polymerase; and (−) torsional stress unwinds and negatively supercoils the DNA behind it (2). In eukaryotic cells, DNA torsional stress is relaxed by topoisomerase I (topo I) and topoisomerase II (topo II) (3,4). Topo I uses the Type-1B mechanism, in which the enzyme temporarily cleaves one strand of the duplex allowing the DNA to rotate around the uncleaved strand (5). Topo II uses the Type-2 mechanism, in which the enzyme removes DNA supercoil crossings by transporting one segment of duplex DNA through a transient double-strand break in another segment (5).

Numerous studies had indicated that DNA relaxation activities of topo I and topo II can substitute each other to support DNA transcription. In particular, relaxation of (+) torsional stress by either topo I or topo II is sufficient to allow the progression of transcribing RNA polymerases (6–8). Accordingly, yeast Δ*top1* single mutants are viable and show no major defects in gene expression (9–11). Similarly, inactivation of topo II does not preclude RNA synthesis in *Saccharomyces cerevisiae* (11), in *Schizosaccharomyces pombe* (12) or in human cells (13). Only when both topoisomerases are defective in yeast *Δtop1 top2-ts* double mutants, global RNA synthesis is reduced (9,14,15).

Other studies, however, have suggested that the DNA relaxation mechanisms of topo I and topo II may not be interchangeable. Yeast minichromosomes with a high (+) DNA torsional stress are efficiently relaxed by topo II but not by topo I (16). Likewise, a recent study indicated that topo I and topo II are prone to relax, respectively, the (−) and (+) torsional constrains produced by the high transcription rate of yeast rDNA genes (17). These distinct preferences of topo I and II had been explained in terms of how torsional stress affects twist (double helical winding) and writhe (supercoiling) deformations of chromatinized DNA (18–20). Chromatin under (+) torsional stress would mainly deform DNA by writhe, which obstructs the strand-rotation mechanism of topo I but facilitates the DNA cross-inversion mechanism of topo II (16). Conversely, chromatin under (−) torsional stress would promote mainly DNA untwisting, which is efficiently relaxed by topo I (17).

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Despite the above observations, however, there is no clear evidence that topo I and topo II play distinctive DNA relaxation roles during general transcription (i.e. in Pol II transcribed genes). One reason is that, in addition to transcriptional elongation, topoisomerases are implicated in the regulation of gene expression at multiple stages. Topo I controls transcription initiation in a subset of yeast genes (10) and facilitates nucleosome disassembly at gene promoters (12). Topo II modulates chromosome architecture and long-range chromatin structure (21); and mainly interacts with gene promoter regions in yeast (11), where it binds nucleosome free DNA and acts redundantly with topo I to enhance recruitment of Pol II (15). Hence, the molecular mechanisms which lead to alterations of DNA transcription on inhibition of cellular topoisomerases are complex; and ascertaining in which steps topo I and topo II are redundant or play specific roles is difficult. Here, we show that the sole inactivation of topo II in budding yeast produces an abrupt decrease of all Pol II transcripts of length above ~3 kb, which is caused by a stalling of Pol II during elongation. Our experiments indicate that this length dependent effect is consequent to the unique capability of topo II, but not topo I, to relax the (+) supercoiled chromatin that accumulates after transcribing a critical DNA length. These findings demonstrate that the DNA relaxation activities of topo I and topo II are not redundant during Pol II transcription *in vivo*. We propose a model to explain why normal production of long gene transcripts requires the topo II mechanism.

MATERIALS AND METHODS

Yeast strains and growth conditions

Saccharomyces cerevisiae strains JCW25 (*TOP1 TOP2*), JCW26 (*TOP1 top2–4*) and JCW28 (*Δtop1 top2–4*) are derivatives of FY251 and had been previously described (22). Yeast strains were grown at 26°C to logarithmic phase in YPD medium containing 2% glucose. At time 0 min, aliquots of the cultures were taken. The rest of the cultures were placed at 37°C and samples were taken after 120 min. Cells were pelleted and immediately stored at −80°C.

RNA preparation

Yeast cells were washed twice by centrifugation (5000*g* at 4°C) in MilliQ water. Total RNA was extracted with the RiboPure Yeast kit (Ambion, Austin, TX, USA) and treated with DNase I (F. Hoffmann-La Roche, Basel, Switzerland) to remove contaminating genomic DNA. Resulting RNA was quantified by spectrophotometry in a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and its integrity checked by gel electrophoresis. Purified RNA was aliquoted and kept at −80°C.

DNA microarray hybridization

Fifteen micrograms of total RNA were used for cDNA synthesis and labeling with Cy3-dUTP and Cy5-dUTP fluorescent nucleotides, following indirect labeling protocol (CyScribe post-labeling kit, GE-Healthcare, New York, NY, USA). Labeling efficiency was evaluated by measuring Cy3 or Cy5 absorbance in a Nanodrop Spectrophotometer. Microarrays encompassing the complete set of 6306 ORFs coded by the *S. cerevisiae* genome, printed with a duplicated set of synthetic oligonucleotides (70-mer, Yeast Genome Oligo Set, OPERON, Cologne, Germany), were provided by the Genomics Unit of the Scientific Park of Madrid (Spain). Microarray prehybridization was performed in 5× SSC (SSC: 150 mM NaCl, 15 mM Na-citrate, pH 7.0), 0.1% SDS, 1% BSA at 42°C for 45 min. (Fluka, Sigma-Aldrich, Buchs SG, Switzerland). Labeled cDNA was dried in a vacuum trap and used as a probe after resuspension in 110 µl of hybridization solution (50% Formamide, 5× SSC, 0.1% SDS, 100 µg/ml salmon sperm from Invitrogen, Carlsbad, CA, USA). Hybridization and washing were performed in a Lucidea Slide Pro System (GE Healthcare, Uppsala, Sweden). Arrays were scanned with a GenePix 4000B fluorescence scanner and analyzed by Genepix® Pro 6.0 software (Axon Instruments, MDS Analytical Technologies, Toronto, Canada). Data was filtered according to spot quality. Only those spots with intensities at least twice the background signal and with at least 75% of pixels with intensities above background plus 2 SDs were selected for further calculations. Following these criteria, over 50% of spots in each array were usually found suitable for further analysis. Genomic data sets for transcript abundance analyses are stored in the GEO databases (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE18242.

Data analysis

Microarray data were analyzed by comparing pairs of a mutant versus reference *TOP1 TOP2* strain. Results for each gene were given as a ratio of pixel intensities (ratio of medians of the mutant strain divided by the reference strain). Ratios were normalized within the Genepix® Pro 6.0 software. The experimental design provided for each condition (0, 120 min) up to six determinations for each gene (three biological replicates and two replicated spots). Those genes for which a minimum of 12 (out of 18) data values passed the microarray quality standards were considered for statistical analyses. Data were calculated as binary logarithms ($\log_2$) of fluorescence ratios. Significant changes of expression values between the starting point and 120 min after the temperature shift were determined by one-way ANOVA ($P < 10^{-3}$).

Quantitative real time PCR analysis

An aliquot of RNA used in the microarray experiments was reserved for qRT-PCR follow-up studies. First strand cDNA was synthesized from 2 µg of total DNAseI-treated RNA in a 20-µl reaction volume using Omniscript RT Kit (Qiagen, Valencia, CA, USA) following manufacture's instructions. qRT-PCR reactions were conducted in triplicate using the SYBR Green PCR Master Mix (Applied Biosystems) and the ABI-PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Gene-specific primers were designed using Primer Express software (Applied Biosystems). Amplified fragments were confirmed by sequencing in a 3730 DNA Analyzer (Applied Biosystems) and comparison with the

published genomic data at SGD. Real time PCR conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of a two-step amplification protocol: denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

Primers used for qRT-PCR are listed in Supplementary Tables S2 and S3.

Genome-wide transcription run-on

Yeast transcription run-on, array hybridization and data analyses were done as previously described (23) with minor modifications. Around 5×10^8 cells were washed in cold water and permeabilized with 0.5% *N*-lauryl sarcosine sodium sulfate. *In vivo* transcription was conducted by incubating cells during 5 min at 30°C in 250 ml of buffer T (20 mM Tris–HCl pH 7.7, 200 mM KCl, 50 mM $MgCl_2$, 2 mM DTT) plus 4 mM each of CTP, ATP, GTP and 12 ml of ^{33}P-UTP (3000 Ci/mmol, 10 mCi/ml). *In vivo* labeled RNA was purified and hybridized ($0.2–2 \times 10^7$ dpm/5 ml) on array membranes. Genomic DNA was random priming labeled and hybridized on the array membranes of the same lot. Genomic data sets for transcription run-on analyses are stored in the GEO databases (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE16673.

Chromatin immunoprecipitation

ChIP experiments for RNA polymerase II were performed as previously described (24) with minor modifications. Briefly, 50 ml of yeast culture were collected at OD_{600} 0.5. Crosslinking was performed by adding formaldehyde to 1% to the culture and by incubating at room temperature for 15 min. Then, 2.5 ml 2.5 M glycine were added and the culture was incubated for 5 min. Cells were then harvested and washed four times with 25 ml Tris–saline buffer (150 mM NaCl 20 mM Tris–HCl pH 7.5) at 4°C. Cell breakage was performed in 300 ml of lysis buffer (see the above reference) with glass beads, and cell extracts were sonicated in a Bioruptor sonicator (Diagenode) for 30 min in 30 s on/30 s off cycles (chromatin is sheared into an average size of 300 bp). Immunoprecipitation was performed with magnetic beads coated with pan anti-IgG antibodies (Dynal), which were incubated before with anti-RNA polymerase II monoclonal antibody 8WG16. Finally, qRT-PCR were performed to quantify immunoprecipitation using a dilution of 1:1500 for the input samples and another of 1:10 for the immunoprecipitated samples. The primers used are listed in Supplementary Table S3.

Extrachromosomal expression of topoisomerases

Topoisomerase expression plasmids YEpTOP2-GAL1, pRK-G1T1 and YEptopA-GAL1, respectively, carry the genes to produce *S. cerevisiae* topo II, *S. cerevisiae* topo I and *Escherichia coli* topo I under the galactose inducible Gal1 promoter (25). Plasmid YEpTOP2 (Y782F)-GAL1 is a derivate of YEpTOP2-GAL1 and induces the expression of a catalytically inactive topo II (26). Plasmid pSTS77 induces the expression of a GyrB-GyrA fusion protein that is catalytically active in *S. cerevisiae* (27). Control plasmid was Yep24 (NEB). Extrachromosomal expression of topoisomerases was induced during 12 h by growing the yeast transformants in selective media containing 1.5% glucose 0.5% galactose for *TOP2* genes, 1% glucose 1% galactose for the *TOP1* gene and 2% galactose for the *TopA* gene and the GyrB-GyrB fusion protein.

RESULTS

Transcriptome response to inactivation of Topo II

Total RNA was extracted from isogenic *TOP2* and *top2-ts S. cerevisiae* strains, before and after shifting the cells to the non-permissive temperature, to inactivate topo II in the mutant strain (37°C). Microarray comparison of polyA+ RNA levels between both strains indicated that topo II inactivation results in relatively minor changes in the yeast transcriptome. After 120 min at 37°C, the relative abundance of 176 transcripts increased and 195 decreased to significant levels (Supplementary Table S1). Gene ontology analysis showed that a large number (29%) of increased transcripts corresponded to genes of ribonucleoproteins involved in ribosome biogenesis, whereas a higher proportion of reduced transcripts (39%) corresponded to genes involved in oxidative metabolism in the mitochondrion (Figure 1A and Supplementary Table S1). Independently from the functional effects observed after the inactivation of topo II, we noticed that nearly all transcripts >3 kb (about 500, representing nearly 9% of the yeast genome) were reduced irrespective of their function (Figure 1A, red diamonds). This drop in abundance of long transcripts was unrelated to temperature changes, as the same transcripts appear essentially unchanged after both 25 to 37°C and 37 to 25°C thermal shifts (28). The decrease of long transcripts abundance was also unrelated to the expression level of their corresponding genes. Both highly and poorly transcribed genes were similarly influenced by the length dependent effect of topo II inactivation (Figure 1B, red diamonds).

Inactivation of Topo II reduces the abundance of long transcripts

A detailed analysis of transcript level variation according to the gene size revealed that average abundance values do not change for transcripts <2.5 kb, an incipient reduction appears for sizes >2.5 kb and an abrupt decrease occurs when transcripts are >3.5 kb (Figure 2A). This length dependence effect was validated by qRT-PCR. Transcript variation of 16 genes of different sizes (Supplementary Table S2) showed a good correlation with the microarray data (Figure 2B); thus confirming that topo II inactivation selectively decreases the production of long gene transcripts.

We next asked if a similar dependence between transcript abundance and their physical length occurred in other topoisomerase mutants. We examined the transcriptome alterations reported for *Δtop1* single mutants (10,29) and for *Δtop1 top2-ts* double mutants (15,29). The alterations of transcript levels in *Δtop1* single mutants were clearly independent of gene size (Figure 2C). In the case of *Δtop1 top2-ts* double mutant, simultaneous inactivation of topo I and topo II produced a global decrease of transcripts abundance relative to the control cells, in

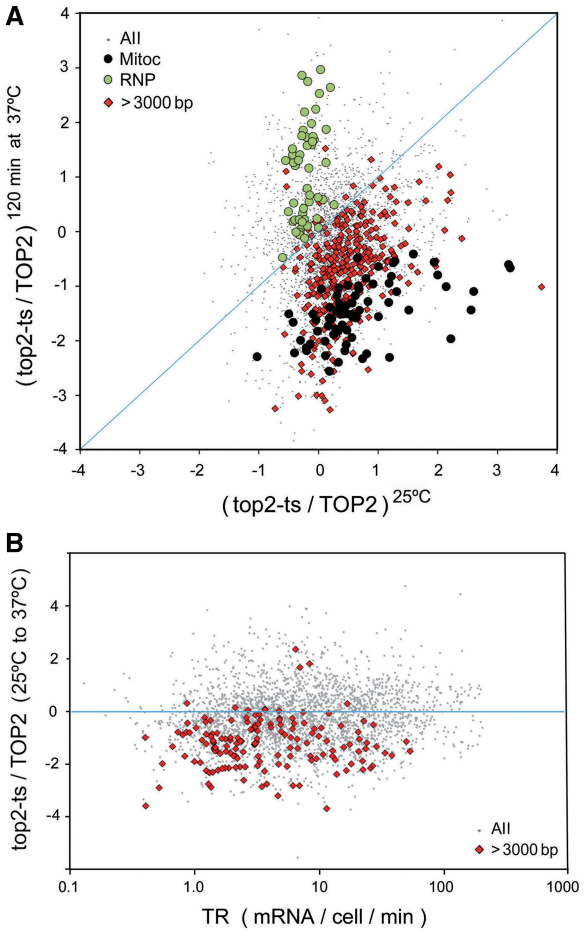

Figure 1. Yeast transcriptome response to the inactivation of topo II. (**A**) Comparison of genome-wide transcript abundance before and after shifting the control *TOP2* strain and the *top2-ts* yeast mutant to the non-permissive temperature. The microarray data is plotted as the ratio [*top2-ts*/*TOP2*] at 25°C (*x*-axis) against [*top2-ts*/*TOP2*] 120 min at 37°C (*y*-axis). Each grey spot corresponds to an ORF. Mitochondrial- and ribonucleoprotein complex-related genes that significantly changed after topo II inactivation are represented by black and green dots, respectively. Red diamonds correspond to yeast transcripts >3 kb. **(B)** The microarray data (*y*-axis), represented as ([*top2-ts*/*TOP2*] at 37°C/[*top2-ts*)/*TOP2*] at 25°C), is plotted against the expression level of yeast genes reported by García-Martinez *et al.* (23). Expression level of each gene (TR, transcription rate) is represented as molecules of mRNA produced/cell/min (*x*-axis, logarithmic scale). Each grey spot corresponds to a gene. Red diamonds correspond to genes >3 kb.

agreement with that observed in previous studies (9,14,15) (Figure 2D). However, this general reduction did not show significant dependence on the transcript length as compared to the selective reduction of long transcripts produced on inactivation of topo II in the *top2-ts* single mutant (Figure 2E).

Inactivation of Topo II does not affect the transcription initiation of long genes

Next, we asked if the reduction of long transcripts abundance caused by topo II inactivation was a consequence of a decrease in their transcription initiation, which is exercised mainly at the RNA polymerase recruitment step and can be determined by transcription run-on (23,30). Therefore, we conducted a genomic transcription run-on (GRO) to examine the variation of transcription rates produced in the *top2-ts* relative to *TOP2* cells. After shifting the yeast cultures to the non-permissive temperature (37°C), yeast cells were permeabilized in a cold sarkosyl buffer that disrupts most chromatin proteins but not the transcribing RNA polymerases. Radioactive UTP was subsequently added to the cells and became incorporated into nascent RNA molecules, which can elongate up to 300 nt in these conditions (23,30). The resulting radiolabeled RNA was extracted and analyzed by hybridization on genomic macro-arrays to provide a measure of the transcription rate of each gene at the moment of cell sampling. The GRO results clearly indicated that genome-wide alterations of transcription initiation produced by the inactivation of topo II are independent of gene size (Figure 3). Therefore, the decrease of long transcript abundance caused by topo II deficiency was not attributable to a failure of RNA polymerase recruitment and initiation of transcription.

Inactivation of Topo II produces a stall in the progression of RNA polymerase II in long genes

Following the above, we examined if the reduction of long transcripts abundance was caused by an impairment of transcription elongation. To do so, we carried out chromatin immunoprecipitation (ChIP) to examine the intragenic distribution of RNA polymerase II (Pol II) in a subset of genes ranging between 1 and 8 kb in length (Supplementary Table S3). Pol II distribution in the *top2-ts* mutant relative to *TOP2* strain after shifting the cells to 37°C produced a similar transcript length dependent pattern in all cases (Figure 4). Irrespective of gene size, inactivation of topo II did not significantly change Pol II allocation up to a 2-kb distance from the transcription start site (TSS). However, as seen in long genes, Pol II markedly accumulated in the intragenic segments between 2 and 3 kb from the TSS; and became depleted in the distal segment beyond 4 kb from the TSS. These results were consistent with the above GRO experiments indicating that topo II inactivation does not alter transcription initiation of long genes; and clearly indicated that the selective reduction of their transcripts was due to an impairment of the progression of Pol II after transcribing between 2 and 3 kb from the TSS.

The effect of Topo II inactivation is not compensated by Type-1 topoisomerases but by relaxing (+) DNA supercoils

Finally, we examined if the reduction of long transcript abundance observed in the *top2-ts* single mutant was solely due to the inactivation of topo II and dependent on its DNA relaxation activity (Figure 5). To do this, we transformed the *TOP2* and the *top2-ts* yeast strains with plasmids Yep24 (control); YEpTOP2-GAL1, which allows ectopic expression of yeast *TOP2* gene under the inducible Gal1 promoter; or its derivative YEpTOP2 (Y782F)-GAL1, which induces the expression of a mutated topo II enzyme that lacks the active-site tyrosine required for catalytic activity (26). On induction

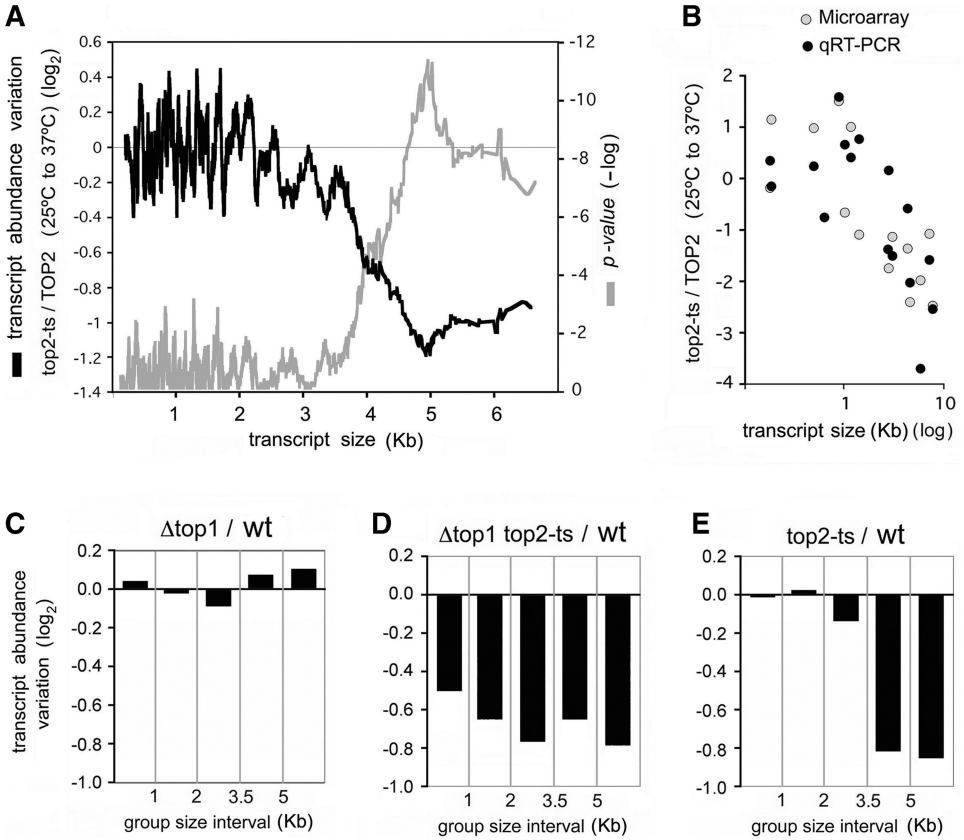

Figure 2. Dependence of transcript abundance and length on inactivation of topo II. (**A**) Microarray data ([*top2-ts*/*TOP2*] at 37°C/[*top2-ts*)/*TOP2*] at 25°C) are represented as sliding means (log_2) of 50 consecutive genes arranged by their size in bp (black line, left scale). *P*-values (log) associated to Student's *t*-test of each point value compared to the whole genome values are illustrated (grey line, right scale). (**B**) Comparison between microarray data (grey dots) and qRT-PCR data (black dots) for 16 genes of different sizes (from 179 to 8018 bp). Results are given as ([*top2-ts*/*TOP2*] at 37°C/[*top2-ts*)/*TOP2*] at 25°C) ratios plotted against gene size (logarithmic scale). (**C–E**) Relative abundance of yeast transcripts grouped by size categories in yeast cells deprived of topo I (*Δtop1 TOP2*), both topo I and topo II (*Δtop top2-ts*), and only topo II (*TOP1 top2-ts*) in comparison to the *TOP1 TOP2* cells (wt).

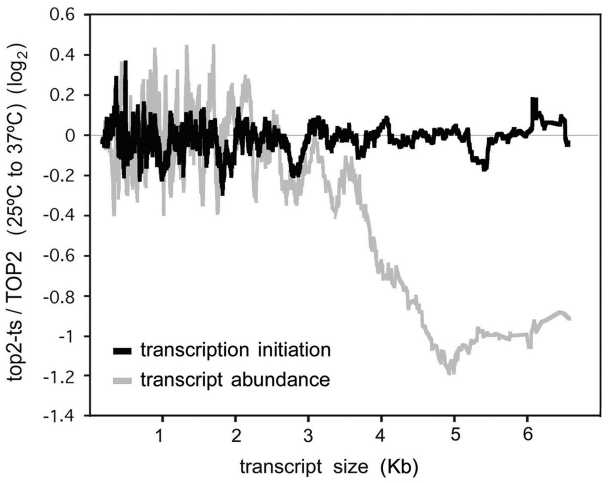

Figure 3. Effects of topo II inactivation on transcription initiation according to gene size. Microarray data from genomic run-on experiments ([*top2-ts*/*TOP2*] at 37°C/[*top2-ts*)/*TOP2*] at 25°C) are represented as sliding means (log_2) of 50 consecutive genes arranged by their size in bp (black line). For comparison, the alterations of transcript abundance observed in the same experimental conditions (as described in the analogous plot of Figure 2A) are shown (grey line).

of the ectopic *TOP2* gene, the reduction of long transcript abundance did not occur in the *top2-ts* cells when shifted to 37°C (Figure 5B). However, the reduction persisted when we induced the topo II mutant instead, thus confirming the requirement of catalytically active topo II as the only cause of the length dependent effect (Figure 5C).

We then conducted analogous experiments to examine if the reduction of long transcripts caused by the lack of topo II activity could be compensated by the ectopic expression of other topoisomerases. We transformed the *TOP2* and the *top2-ts* mutant strains with plasmid pRK-G1T1 to overproduce *S. cerevisiae* topo I; with plasmid YEp*topA*-GAL1 to produce *E. coli* topo I (23) and with plasmid pSTS77 to produce an *E. coli* GyrB-GyrA fusion protein (27). As illustrated in Figure 5, the yeast topo I uses a DNA strand-rotation mechanism to relax (+) and (−) torsional stress (3,5). The bacterial topo I uses a DNA single-strand-passage mechanism on unwound regions of the duplex and relaxes (−) torsional stress when it is produced in yeast cells (25). The GyrB-GyrA fusion protein (GyrBA) uses a DNA cross-inversion mechanism like topo II and relaxes (+) torsional stress when it is produced in yeast (27).

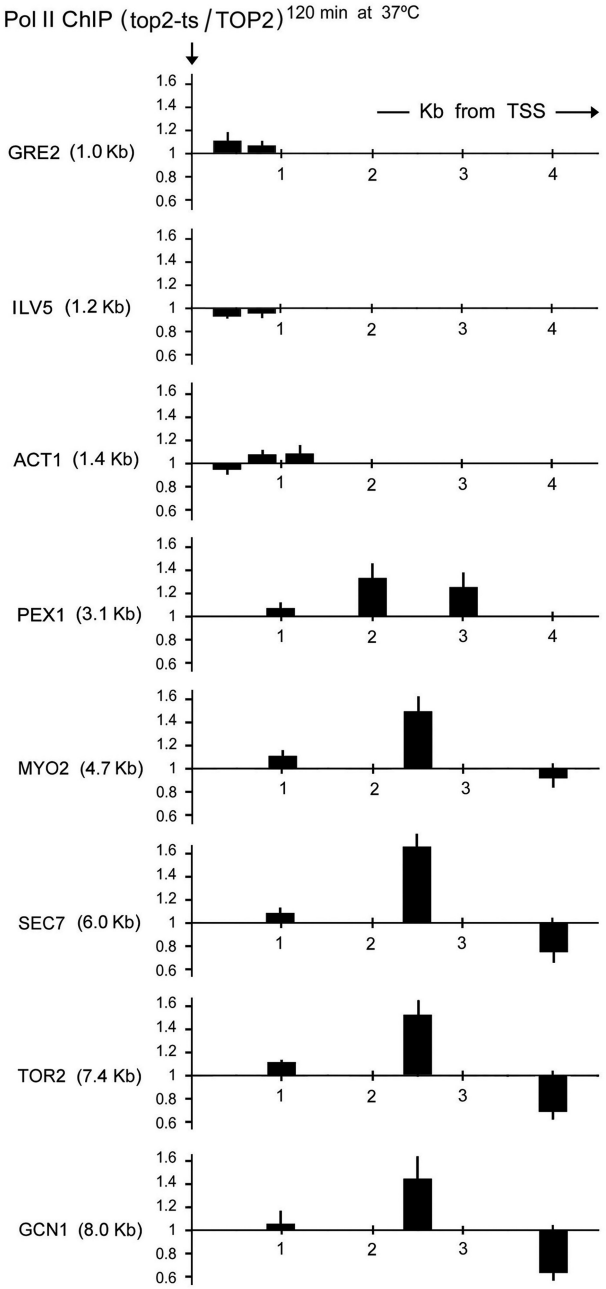

Figure 4. Changes in the intragenic distribution of RNA polymerase II after topo II inactivation. Pol II ChIP DNA fragments (average length ~350 bp) located at increasing distances from the transcription start site (TSS) were quantified by qRT-PCR in eight yeast genes (1.0 kb GRE2; 1.2 kb ILV5; 1.4 kb ACT1; 3.1 kb PEX1; 4.7 kb MYO2; 6.0 kb SEC7; 7.4 kb TOR2; 8.0 kb GCN1). The positions of the intragenic fragments analyzed are illustrated for each individual gene. Histograms show the ratio of RNA pol II density (left scale) of the *top2-ts* relative to the control *TOP2* yeast strain following a 120-min shift to 37°C. The average of three experiments is shown. Error bars represent the SD.

These compensation experiments showed that the ectopic production of yeast topo I (Figure 5D) and bacterial topo I (Figure 5E) in the *top2-ts* cells did not prevent the reduction of long transcript abundance on inactivation of topo II. However, the expression of the GyrBA enzyme was able to compensate the absence of topo II activity (Figure 5F). Therefore, the incapacity to relax (+) supercoils by topo II, but not topo I, was the more likely cause of the stall of Pol II and consequent reduction of long gene transcripts.

DISCUSSION

Whether the DNA relaxation activities of eukaryotic topo I and topo II are redundant or have distinctive roles during genome transactions is a long-standing question. In this study, we show that the sole inactivation of topo II in *S. cerevisiae* produces a decrease of virtually all Pol II transcripts of length above ~3 kb, irrespective of their function. This selective reduction is not due to an alteration of transcription initiation, but to an impairment of the RNA polymerase during elongation. Therefore, proper production of this subset of transcripts demands a specific role of topo II, which cannot be compensated for by topo I.

Any dependence of transcript abundance on transcript length can be reasoned by the alleged role of topoisomerases in removing the DNA torsional stress generated during transcription elongation (3,4). *In vitro* studies have shown that the failure to relax (+) torsional stress stalls the progression of transcribing polymerases (6,7). The same has been concluded from recent *in vivo* work, which showed that convergent transcription is impaired under topo I and topo II inactivation as a function of supercoiling accumulation (8). Hence, a dependence on transcript length is expected in cells deficient in topoisomerase activity, since the longer the transcribed distance the more (+) torsional stress will be accumulated. Strikingly, we do not observe such length dependence in *Δtop1 top2-ts* double mutants of *S. cerevisiae*. Inactivation of both topoisomerases causes instead a general reduction of transcript abundance, which probably reflects a global down-regulation of transcription by alterations in both the initiation and elongation steps, irrespective of the gene size (12,15,29). Our observation is consistent with a recently reported intragenic distribution of Pol II in *Δtop1 top2-ts* double mutants of *S. cerevisiae,* which showed no significant length dependence alterations relative to wild-type strains (15). Similar results have been found for *Δtop1 top2-ts* double mutants of *S. pombe*; in which only a slight accumulation of Pol II was observed in coding regions of genes (12).

Contrasting with the above, the transcript length dependence uncovered here is prominent and occurs specifically in *top2-ts* single mutants. No such dependence is observed in *Δtop1* single mutants. The inactivation of topo II does not affect the transcription frequency of long genes. It produces a stall in the advancement of Pol II after transcribing beyond 2 kb, which suitably explains the selective decrease of long transcript abundance. The transcript reduction is not rescued by overproducing yeast topo I or bacterial topo I, which relaxes (−) DNA supercoils. It is rescued by catalytically active topo II or a GyrBA enzyme, which relaxes (+) DNA supercoils. Therefore, the more likely cause of the stall of Pol II and subsequent reduction of long transcripts is the incapacity of the *top2* cells to relax the (+) DNA supercoils

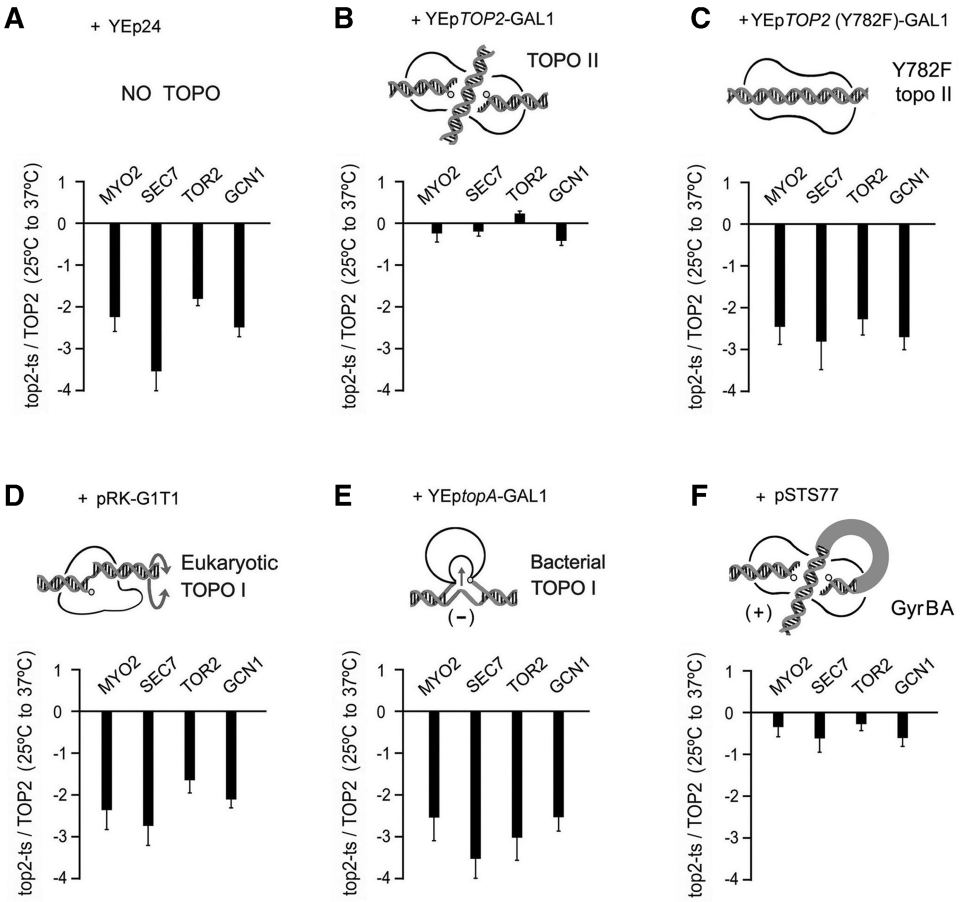

Figure 5. Effect of the ectopic expression of topoisomerases on the abundance of long transcripts. *TOP2* and *top2-ts* yeast strains were transformed with plasmids that carried distinct DNA topoisomerase genes under the galactose inducible Gal1 promoter: (**A**) Yep24 (control), (**B**) YEpTOP2-GAL1 (*S. cerevisiae* topo II), (**C**) YEpTOP2 (Y782F)-GAL1 (catalytically inactive *S. cerevisiae* topo II), (**D**) pRK-G1T1 (*S. cerevisiae* topo I), (**E**) YEp*topA*-GAL1 (*E.coli* topo I) and (**F**) pSTS77 (a GyrB-GyrA fusion protein). Induction of the ectopic genes was done during 12 h before shifting the yeast cultures to 37°C to inactivate cellular topo II. The variations of transcript abundance of four genes (4 kb MYO2; 6.0 kb SEC7; 7.4 kb TOR2; 8.0 kb GCN1) were examined by qRT-PCR. Results are given as ([*top2-ts*/*TOP2*] at 37°C/[*top2-ts*)/*TOP2*] at 25°C) ratios. The average of three experiments is shown. Error bars represent the SD. As explained in the main text, the distinct DNA relaxation mechanisms of the expressed topoisomerases are illustrated.

accumulated during transcription elongation. This finding substantiates the biological relevance of what we had observed in previous *in vitro* studies: (+) supercoiled chromatin is efficiently relaxed by the DNA cross-inversion mechanism of topo II, but not by the DNA strand-rotation mechanism of topo I (16).

An intriguing aspect of the transcript length dependence exposed here is that, rather than being gradual, it has a sharp inflection point at ~3 kb. Up to this critical length, (+) torsional stress can be either relaxed by topo I alone or it is not high enough to delay the normal progression of Pol II. Beyond this length, topo I is no longer efficient and topo II is then required to avoid the stalling of Pol II. We believe that this inflection reveals how increasing levels of (+) torsional stress affect the conformation of intracellular chromatin. In this regard, *in vitro* studies had shown that torque generated ahead of RNA polymerases is enough to partially unwrap nucleosomal DNA and flip from (−) to (+) the entry and exit DNA crossing of the nucleosomes (18–20). This plasticity allows eukaryotic chromatin to buffer moderate levels of (+) torsional stress without shortening or buckling supercoils. However, when this buffering capacity is surpassed, chromatin enters into a supercoiling regime (18–20). At this stage, chromatin tightly condenses and (+) torsional stress may enforce also a flip of the global left-handed chirality of nucleosomes (18). Remarkably, we had previously showed that chromatin is efficiently relaxed by topo II but not by topo I when its DNA superhelical density is >0.04 (16), a value in which chromatin has entered the supercoiling regime (18–20). With these premises, the abrupt reduction of long transcript production reported here is likely to reflect the transition of intracellular chromatin from the buffering stage to the supercoiling stage during transcription elongation, as illustrated by the model in Figure 6. This transition probably occurs during normal transcription, since the generation rate of torsional stress in front of RNA polymerases is higher than the relaxation rate provided by cellular topoisomerases (19,31). Accordingly, we propose that topo I activity alone and the buffering

capacity of yeast chromatin are sufficient to support the normal production of transcripts up to ~3 kb in length. After this point, increasing levels of (+) torsional stress drive chromatin into the supercoiling regime downstream of the transcription complex. Now, topo I is no longer efficient and the relaxation of DNA by topo II activity becomes essential to support the normal progression of the RNA polymerase.

Our conclusions on the requirement of topo II activity during Pol II elongation are comparable to those inferred for Pol I transcription of yeast rDNA genes. Former studies indicated that inactivation of either topo I or topo II stimulates Pol I transcription initiation; whereas inactivation of both enzymes affected the elongation of long, but not short, Pol I transcripts (14). More recent visualization analyses revealed that effective elongation by Pol I depends mainly on topo II activity (17). Therefore, although Pol I and Pol II markedly differ in the structure and dynamics of their transcribing ensembles, topo II may be similarly required for the removal of (+) DNA supercoils accumulated ahead of both RNA polymerases. The biological relevance of this activity is probably masked by the other essential roles of topo II during chromosome replication and segregation (4,21). Since topo II inactivation decreases the abundance of long transcripts but does not abolish them, incipient or minor phenotypes are possibly undetected along the other major or lethal effects of topo II.

In prokaryotes, DNA topoisomerases have a clear division of roles to relax DNA during transcriptional elongation. Bacterial topo I specifically relieves (−) torsional stress, whereas DNA gyrase removes (+) supercoils (3,4). In eukaryotes, however, since topo I and topo II are able to relax both (+) and (−) torsional stress, a general belief is that both enzymes can substitute for each other in these tasks. Our study demonstrates that this is not the

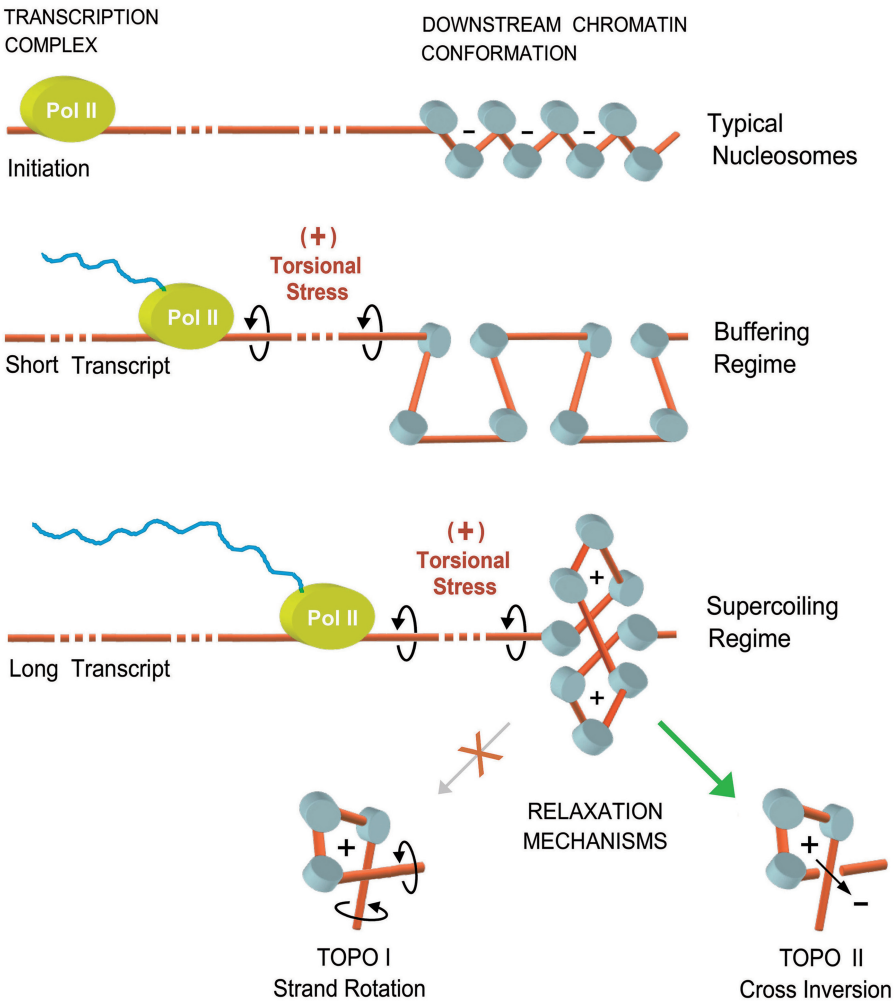

Figure 6. Model to explain the specific role of topo II during transcription elongation in long genes. At transcription initiation, the typical nucleosome organization of downstream chromatin stabilizes (−) DNA supercoils. During transcriptional elongation, (+) torsion builds up in the DNA in front of the RNA polymerase. Because its generation rate exceeds its relaxation rate by cellular topoisomerases, (+) torsional stress increases in a transcript-length dependent manner. This torsional stress diffuses to downstream regions, where it is buffered by alterations in the topology of nucleosomal DNA. When this buffering capacity is surpassed, downstream chromatin enters the supercoiling regime. In this chromatin conformation, the DNA strand-rotation mechanism of topo I is not efficient and only the DNA cross-inversion mechanism of topo II is able to remove the (+) DNA supercoils, which would otherwise stall the progression of the RNA polymerase.

case. Topo II has a specific role in removing (+) supercoils accumulated during transcriptional elongation, a function that cannot be compensated by cellular topo I. These findings imply also that biological and pharmacological effects of topoisomerase inhibitors could be dependent on local levels of DNA supercoiling and, thus, on the transcript length of neighbouring genes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3.

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