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Citation for published version:

Digital Object Identifier (DOI):
10.1128/MCB.00589-10

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Molecular and Cellular Biology

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Download date: 30. May. 2021
Distinguishing the Roles of Topoisomerases I and II in Relief of Transcription-Induced Torsional Stress in Yeast rRNA Genes

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Received 21 May 2010/Returned for modification 6 July 2010/Accepted 15 November 2010

Eukaryotic cells have two major topoisomerases that are capable of efficiently relaxing torsionally stressed DNA: topoisomerase I (Top1) and topoisomerase II (Top2) (75). They are both abundant nuclear proteins with roles in many DNA activities, and since they both can relax positive and negative torsion, they can substitute for each other in most situations [11, 28, 29, 35, 62]. In spite of this partial functional redundancy, they control DNA topology by very different mechanisms (65). Top1 (a type IB topoisomerase) makes transient single-strand breaks in torsionally stressed DNA (recognizing the torque in such DNA), followed by controlled rotation of the nicked strand and resealing of the DNA in a more relaxed state (38). Top2 (a type IIA topoisomerase) recognizes juxtaposed DNA helices (as in supercoiled DNA) and passes one DNA helix through the other by making a transient double-strand break in one of the helices (61, 65). Top2 plays an essential role during S phase because it is required to decatenate chromosomes, thus preventing their breakage and loss during cytokinesis (5). Yeast cells without Top1 grow very well, whereas cells lacking functional Top2 remain viable if they are prevented from completing mitosis (5, 11, 28, 29, 70).

To better understand the role of topoisomerase activity in relieving transcription-induced supercoiling, yeast genes encoding rRNA were visualized in cells deficient for either or both of the two major topoisomerases. In the absence of both topoisomerase I (Top1) and topoisomerase II (Top2) activity, processivity was severely impaired and polymerases were unable to transcribe through the 6.7-kb gene. Loss of Top1 resulted in increased negative superhelical density (two to six times the normal value) in a significant subset of rRNA genes, as manifested by regions of DNA template melting. The observed DNA bubbles were not R-loops and did not block polymerase movement, since genes with DNA template melting showed no evidence of slowed elongation. Inactivation of Top2, however, resulted in characteristic signs of slowed elongation in rRNA genes, suggesting that Top2 alleviates transcription-induced positive supercoiling. Together, the data indicate that torsion in front of and behind transcribing polymerase I has different consequences and different resolution. Positive torsion in front of the polymerase induces supercoiling (writhe) and is largely resolved by Top2. Negative torsion behind the polymerase induces DNA strand separation and is largely resolved by Top1.

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Published ahead of print on 22 November 2010.
and can twist the DNA into positive supercoils. In the absence of only Top1, plasmid templates may be subject to high negative torsion when transcribed (12). Negative torsion can be relieved either by negative supercoiling or by unwinding of the DNA duplex, suggesting that the helix might become unwound in short regions behind polymerases (12) as has indeed been shown to occur in the C-myc promoter (39, 40). Studies on *Escherichia coli* strains deficient in topoisomerase (21) have shown that negative torsion is conducive to hybrid formation between underwound template DNA and nascent RNA. Such DNA-RNA hybrids together with the displaced nontemplate DNA strand are called R-loops. R-loops block transcription elongation (32, 71) and in bacteria are deleterious to cell viability (3, 49, 50). We have recently shown that loss of Top1 enhances R-loop formation in yeast rRNA genes concomitant with an increase in transient blocks to elongation (23).

Although many studies have used topoisomerase-deficient cells to study the role of these proteins in transcription (76), most have used reporter genes on plasmid templates. In the present study, the well-characterized rRNA genes are studied in their normal chromosomal context, and for the first time, a distinction between the consequences of Top1 versus Top2 deficiency on active rDNA and support the recent speculation that Top1 plays the major role behind elongating polymerases, while Top2 does so in front of elongating polymerases (42). We also found that top1Δ cells can tolerate large regions of melted rDNA template (up to 2 kb), which is nonetheless compatible with continued transcription of the affected gene, unlike transcriptional R-loops, which block elongation (23, 32, 71). These results provide insight into the abnormal DNA topology and genetic instability found in yeast rDNA in topoisomerase-deficient strains.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions.** Yeast strains were grown in YPD at 30°C unless otherwise indicated. Yeast strains used were control strains BY4741 (MATa his3Δ1 leu2Δ2 ada2Δ0), BY4741 (MATa his3Δ1 leu2Δ2 met15Δ0 top1Δ0) (10), and BY169 (BY4741 but Top1Δ1::HIS3) (the present study). TOP1 deletion strains were SY84 (BY4741 but top1Δ1::KanM4Δ) (79), W1854-2A (W303 but top1Δ1::LEU2 RAD50) (kindly provided by Rodney Rothstein), and YB168 (BY4741 but top1Δ1::KanM4::Top-RFA1::HIS3) (the present study). Control and experimental strains for RNase H under- and overexpression were YAEH21 [BY4741 but F*Cas9-HA-TOP1* (KanM6)], YAEH25 [BY4741 but F*Cas9-HA-TOP1* (KanM6) rsb1Δ], YAEH267 [BY4741 but top1Δ1::HIS3], and YAEH269 [BY4741 but top1Δ1::HIS3] (kindly provided by Rodney Rothstein).

**ChIP.** Chromatin immunoprecipitation (ChIPs) were performed as previously described (19). Exponentially growing cells (optical density at 600 nm [OD₆₀₀ of ~1.0] in 100 ml of YPD medium were cross-linked with 1% formaldehyde for 20 min and then washed twice with 10 ml of cold TBS (300 mM NaCl, 40 mM Tris-HCl [pH 7.5]). The cell pellets were resuspended in 0.6 ml of FA-lys 140 buffer (50 mM HEPES, 140 mM NaCl, 1.0% Triton X-100, 1.0 mM EDTA, 0.1% sodium deoxycholate) containing protease inhibitors and then disrupted in a FastPrep FP120 machine (Bio 101/Termalovasant) at 4°C (shaking 7× for 45 s each time with 1 min on ice in between shakings). The cell extracts were collected into microfuge tubes, sonicated with eight 10-s pulses (30% output, 90% duty cycle) on ice, and then centrifuged at 14,000 rpm in an Eppendorf microcentrifuge for 5 min at 4°C. The supernatants were transferred to a new microfuge tube, and the protein content was quantified by using a Bradford assay (Bio-Rad). For ChIP immunoprecipitations (IPs), equal protein content (1.25 to 1.5 mg) per extract was incubated in 0.6-ml microfuge tubes with FA-lys 140 solution in a total volume of 400 µl. For the input chromatin controls, 1/10 of the extract volume used for IP was processed independently without antibody addition. An anti-protein A antibody was added to the IP samples (Sigma; 1:50,000 dilution), and the tubes were rotated overnight at 4°C. One-tenth of the chromatin extract volume used for IP was used as the input control extract. The IP supernatants were added to new 0.6-microfuge tubes containing 60 µl of protein A-Sepharose beads (50% slurry in FA-lys 140 buffer) and rotated at 4°C for 4 h. Beads were then washed twice with 0.5 ml of FA-lys 140 buffer, four times with 0.5 ml of FA-lys 500 buffer (50 mM HEPES, 500 mM NaCl, 1.0% Triton X-100, 1.0 mM EDTA, 0.1% sodium deoxycholate), and four times with 0.5 ml of LiCl detergent wash buffer (10 mM Tris-HCl [pH 8.0], 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate). The immune complexes were precipitated twice with 75 µl of elution buffer (5% TE + 1% sodium dodecyl sulfate) and then incubated overnight at 65°C to reverse the cross-links. DNA was purified by using a PCR purification kit (Qiagen). PCR conditions were as follows: 92°C for 3 min (1 cycle); 92°C for 30 s, 52°C for 30 s, and 72°C for 45 s (22 cycles for rDNA or 29 cycles for 4.3 SAATGCGGCAGATGCGTCTTCCTCC-3’), rDNA2 (positions 1061 to 1274, 5’-CGGGAATTCATCAGTGCTGCTCC-3’), rDNA3 (positions 1061 to 1274, 5’-CGGGAATTCATCAGTGCTGCTCC-3’), rDNA4 (positions 3001 to 3214, 5’-CGGGAATTCATCAGTGCTGCTCC-3’), rDNA5 (positions 3001 to 3214, 5’-CGGGAATTCATCAGTGCTGCTCC-3’), rDNA6 (positions 3001 to 3214, 5’-CGGGAATTCATCAGTGCTGCTCC-3’), rDNA7 (positions 5007 to 5224, 5’-CGGGAATTCATCAGTGCTGCTCC-3’). rDNA8 (positions 5007 to 5224, 5’-CGGGAATTCATCAGTGCTGCTCC-3’), rDNA9 (positions 5007 to 5224, 5’-CGGGAATTCATCAGTGCTGCTCC-3’), rDNA10 (positions 5007 to 5224, 5’-CGGGAATTCATCAGTGCTGCTCC-3’).
RESULTS

Unusual appearance of rRNA genes in top1Δ cells. EM visualization of nucleolar chromatin dispersed by Miller spreading allows quantitative analysis of many rRNA genes from multiple nucleoli (25, 57), thus combining the advantages of studying both individual genes and gene populations. The method was used to analyze rDNA from control yeast strains and strains lacking Top1 (top1Δ). These genes were robustly transcribed in top1Δ cells (Fig. 1A), as expected given the near-normal growth rate of top1Δ. In a companion study, we found that RNA genes from top1Δ had, on average, somewhat more polymerases per gene compared to the control strain (examples in Fig. 1B) and more polymerase pileups than the average, with topo-bubbles in some but not all of the polymerase-free gaps (middle gene). The lower gene has high pol density with a small bubble in a small polymerase-free gap. Bubbles 8 to 10 are enlarged at bottom.

rDNA8 (positions 6607 to 6825), 5'-GTTACGATCTCTGAGATTAAGC-3' and 3'-GGTACGATCTGAGATTAAGC-3'; NTS1 (positions ~2189 to ~1912), 5'-GAAAGGAATTGCGCCGGAGCTTG-3' and 3'-GAAAGGAATTGCGCCGGAGCTTG-3'; NTS2 (positions ~265 to ~260), 5'-GTAGTTTTGTATGTTCCCG-3' and 3'-CATGAAGTACCTCCCAACTAATTC-3'; and ACT1, 5'-CCCCATTTTCTGAGAGATTTTG-3' and 3'-CCCCATTTTCTGAGAGATTTTG-3'.

Topo-bubbles in rDNA in top1Δ cells are not replication bubbles. Newly initiated DNA replication events also appear as bubbles; we sought to determine whether these could be distinguished from topo-bubbles in the rDNA. There is an origin of replication in each nontranscribed spacer (NTS) in all ~150 rDNA repeats (r-ARS in Fig. 2A); ~20% of these fire during each S phase (45). Replication bubbles seen shortly after firing occurred at the expected position in the NTS (63), shown in the upper EM image in Fig. 2A from top1Δ cells. For comparison, a topo-bubble in the 5' region of an rRNA gene is shown in the lower EM image in Fig. 2A (enlarged image in inset). A set of neighboring replication and topo-bubbles is shown in the upper EM image in Fig. 2A (enlarged image in inset). The two types of bubbles differed by several criteria. First, replication bubbles typically had a particle at each end, corresponding to the replication machinery, whereas topo-bubbles were only rarely bracketed by particles, which could be identified as RNA polymerases (see below). Second, topo-bubbles were within the active genes, whereas short replication bubbles originated in the NTS. Third, replication bubbles ranged widely in length, from very small to 9 µm, corresponding to 27 to 30 kb or ~3 rDNA repeats (Fig. 2D). Topo-bubbles were on average much shorter, 0.12 µm or

FIG. 1. rRNA genes in top1Δ cells are robustly transcribed and display unusual bubble structures in the active rDNA template. (A) The top panel shows an EM image of ~20 active rRNA genes from a single yeast nucleolus from top1Δ cells (bar, 0.5 µm). Numbered arrows point to bubble structures in genes. Bottom panel shows each bubble at a higher magnification with an accompanying trace of the DNA strand in which the bubble occurs. (B) Representative rRNA genes from the WT and top1Δ (SY84) strains, with each gene displaying ~average polymerase density for the strain. Bars (for genes in panels B and C), 0.5 µm. (C) Single rRNA genes from top1Δ cells. The genes in the two panels on top have fewer polymerases than average, with topo-bubbles in some but not all of the polymerase-free gaps (middle gene). The lower gene has high pol density with a small bubble in a small polymerase-free gap. Bubbles 8 to 10 are enlarged at bottom.
~420 nt, with the largest seen being ~2.1 kb (Fig. 2D and Table 1). Lastly, multiple topo-bubbles (two to four) occurred in 27% of bubble-containing rRNA genes, whereas only a single replication origin is present per repeat. We conclude that the DNA bubbles seen in active genes in top1 strains are not replication intermediates. Topo-bubbles are not R-loops, but their appearance is dependent on RNase H activity. Since R-loops might appear as bubbles in transcribed DNA (32), we addressed the relationship of topo-bubbles to transcriptional R-loops. R-loops are increased in DNA that is under negative torsional stress, including rRNA genes in E. coli top4 mutants (21, 50) or in yeast cells lacking both Top1 and RNase H (23). RNase H removes R-loops by digesting the RNA strand of RNA-DNA hybrids (16). If topo-bubbles are R-loops, we anticipated that they would be stabilized when RNase H is inhibited in Top1-depleted strains, leading to increased numbers and/or length of bubbles, and removed when RNase H is overexpressed. This was found not to be the case.

To decrease RNase H activity, it was necessary to inhibit the expression of both RNase H1 and RNase H2, which have overlapping functions (1). In rnh1/rnh201 double-mutant strains, RNase H activity is decreased to a small percentage of normal (1, 23). Growth and rRNA synthesis are not clearly affected, but the combined mutation is synthetic lethal with top1 (23). We analyzed rRNA genes in PGAL-TOP1 rnh1/rnh201 strains following depletion of Top1 by transfer from galactose to glucose medium for 6 h. This is 4 h prior to

<table>
<thead>
<tr>
<th>Strain</th>
<th>Topo-bubble lengtha</th>
<th>No. of bubbles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined top1Δ strains</td>
<td>0.123 (~415 bp)</td>
<td>220</td>
</tr>
<tr>
<td>Individual top1Δ strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY84 (top1Δ)</td>
<td>0.113</td>
<td>84</td>
</tr>
<tr>
<td>W1854-2A (top1Δ)</td>
<td>0.140</td>
<td>71</td>
</tr>
<tr>
<td>YB108 (top1Δ Tap-RFA1)</td>
<td>0.119</td>
<td>20</td>
</tr>
<tr>
<td>YAEH267 (top1Δ)</td>
<td>0.119</td>
<td>45</td>
</tr>
<tr>
<td>W1477-5B (top1Δ top2-ts)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23°C</td>
<td>0.112</td>
<td>106</td>
</tr>
<tr>
<td>35°C</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

a Ranges in parentheses were derived by assuming an average DNA compaction of 3,370 bp per 1 μm of rRNA gene length in EM spreads, which was determined empirically from rRNA genes measured for this study. This is somewhat compacted from B-form DNA (2,940 bp/μm). NA, not applicable because no topo-bubbles were seen in rRNA genes in this strain after inactivation of Top2 at 35°C for 2 to 4 h.

~420 nt,
manifestation of a growth defect and 8 to 10 h prior to growth inhibition (23), thus avoiding any indirect effects of poor growth rate. For comparison, TOP1 expression was similarly repressed for 6 h in a strain expressing wild-type (WT) RNase H levels. As shown in Fig. 3A, topo-bubble frequency decreased by more than 90% in cells deficient for both RNase H and Top1 compared to cells deficient for Top1 only. Analysis of individual rRNA genes revealed 23 bubbles in 376 genes (6.1%) after 6 h depletion of Top1, whereas only 2 of 513 genes displayed bubbles (0.4%) in rnhΔ cells after 6 h Top1 depletion (Fig. 3A; examples in Fig. 3B). In the presence of Top1 (growth in galactose), no topo-bubbles were seen in either strain (not shown). The higher topo-bubble frequency in top1Δ strains (~30% of genes) compared to GAL::TOP1 strains grown for 6 h on glucose (6% of genes) may reflect the different degrees of Top1 depletion.

We sought to determine whether topo-bubbles were reduced upon overexpression of RNase H, as might be expected for R-loops. To increase RNase H activity, RNase H2 was overexpressed by Gal-induction of RNH201, a treatment shown to increase digestion of telomeric R-loops in yeast (48). Western blotting confirmed the robust induction of RNH201 by galactose in strain YAEH269, which is also top1Δ (23). We measured topo-bubble length and frequency in YAEH269 after growth in galactose and compared these parameters to top1Δ cells in the same conditions. There was no significant difference in the frequency (30 versus 31% of genes) or average length (0.12 versus 0.13 μm) of topo-bubbles in top1Δ cells in the presence of endogenous versus overexpressed levels of RNase H (Fig. 3A; examples in Fig. 3C).

Combined data indicate that topo-bubbles are not R-loops, since they were not stabilized when RNase H was deficient and they were not destabilized when RNase H was overexpressed. Rather, efficient formation of topo-bubbles was dependent on RNase H activity (Fig. 3A). We propose that in the absence of Top1, digestion of R-loops is needed to clear blocks to transcription elongation and thus allow the buildup of torsional stress that generates topo-bubbles (see model in Fig. 8).

Evidence that topo-bubbles are regions of DNA helix melting. Since topo-bubbles did not have characteristics of replication bubbles or R-loops, we considered that they might consist of unwound single-stranded DNA. Their appearance in EM images supported this conclusion, since both sides of the topo-bubbles had a similar form, which is expected of single-stranded DNA bubbles but not of R-loops. Also, there was no visible evidence of RNA involvement in the bubbles; that is, no RNA attached to a nearby polymerase could be seen feeding into them, and no free RNA tails emerged (Fig. 4A). The presumed single-stranded DNA within the topo-bubbles gen-

FIG. 3. Effect of RNase H under- and overexpression on topo-bubbles in Top1-deficient cells. (A) The left plot shows the topo-bubble frequency in Top1-deficient cells in conditions of RNase H under- and overexpression. For RNase H underexpression, strain YAEH275 (PGAL-TOP1 rnhΔ) was switched from galactose to glucose medium for 6 h to deplete Top1. Its control strain was YAEH271 (PGAL-TOP1), which was also depleted of Top1 for 6 h. For RNase H overexpression, strain YAEH269 (top1Δ+gAL-RNH201) was grown in galactose medium for expression of RNH201. Its control strain YAEH267 (top1Δ+pgAL) was similarly grown. Topo-bubble frequency in experimental strains (i.e., Top1-deficient and plus or minus RNase H) is shown as a fraction of the frequency in Top1-deficient control strains; (see the text for the frequency of bubble-containing genes). The bar graph on the right shows the average topo-bubble length (error bars indicate the standard deviation) in YAEH267 compared to YAEH269 using same gene populations used for topo-bubble frequency. (B) Representative rRNA genes from RNase H underexpression experiment in panel A, showing examples with (+) and without (−) topo-bubbles from both strains. Topo-bubbles within genes are enlarged in insets (arrows). (C) Same as panel B for RNase H overexpression experiment.
Topo-bubbles appear as regions of DNA helix melting and preferentially occur in AT-rich regions of the gene. (A) Shown at left are portions of four active rRNA genes from top1Δ with topo-bubbles showing typical characteristics: both sides of the bubble appear similar and with no evidence of RNA involvement, even when immediately juxtaposed to RNA polymerases. At the right are portions of two active rRNA genes from top1Δ obtained using modified Miller spreading conditions (KCl concentration increased by 11 mM) (57). In these conditions, DNA in topo-bubbles appears thicker than flanking double-stranded DNA (see the text). Bar, 0.1 μm. (B) Schematic of rDNA repeat at top is aligned with a graph showing the percent AT content across the repeat (middle) and a plot of bubble position across the repeat (bottom). The latter was derived by normalizing gene length to 100 U, dividing each gene into 50 bins of 2 U each, and scoring each bin across the gene as positive or negative (for denatured DNA at that position) for each bubble-containing gene analyzed. n = 101 genes. (C) Gene schematic is aligned with EM of an active rRNA gene with two topo-bubbles (bracketed black arrows). Double-headed red arrows indicate nearest-neighbor RNA polymerases up- and downstream of both bubbles. This gene and 35 additional genes are schematized below the EM image, with each gene normalized in length and with topo-bubble occurrence shown. Nearest-neighbor polymerases up- and downstream of each bubble are shown as red ovals. Shaded bars indicate the three most AT-rich regions, as shown in panel B.

FIG. 4. Topo-bubbles appear as regions of DNA helix melting and preferentially occur in AT-rich regions of the gene. (A) Shown at left are portions of four active rRNA genes from top1Δ with topo-bubbles showing typical characteristics: both sides of the bubble appear similar and with no evidence of RNA involvement, even when immediately juxtaposed to RNA polymerases. At the right are portions of two active rRNA genes from top1Δ obtained using modified Miller spreading conditions (KCl concentration increased by 11 mM) (57). In these conditions, DNA in topo-bubbles appears thicker than flanking double-stranded DNA (see the text). Bar, 0.1 μm. (B) Schematic of rDNA repeat at top is aligned with a graph showing the percent AT content across the repeat (middle) and a plot of bubble position across the repeat (bottom). The latter was derived by normalizing gene length to 100 U, dividing each gene into 50 bins of 2 U each, and scoring each bin across the gene as positive or negative (for denatured DNA at that position) for each bubble-containing gene analyzed. n = 101 genes. (C) Gene schematic is aligned with EM of an active rRNA gene with two topo-bubbles (bracketed black arrows). Double-headed red arrows indicate nearest-neighbor RNA polymerases up- and downstream of both bubbles. This gene and 35 additional genes are schematized below the EM image, with each gene normalized in length and with topo-bubble occurrence shown. Nearest-neighbor polymerases up- and downstream of each bubble are shown as red ovals. Shaded bars indicate the three most AT-rich regions, as shown in panel B.

erally appeared similar to the flanking double-stranded DNA (Fig. 4A, left panel), but typical Miller-spread conditions are known to remove some nucleic-acid-bound proteins. When we used more physiological spreading conditions (56, 57), both strands of DNA within the bubble still appeared similar, but were now thicker than the flanking DNA (Fig. 4A right). Single-stranded DNA forms complexes with ssDNA binding proteins and has been shown to appear thicker than a DNA helix in EM preparations (73).

Mapping of topo-bubble positions revealed that they occurred preferentially in AT-rich regions of the rRNA gene (Fig. 4B and C). AT-rich helices have fewer hydrogen bonds than GC-rich helices and thus are more susceptible to thermal and torsional denaturation (54). Bubble positions were mapped for 101 genes from four different top1Δ strains with similar results; combined data are shown in Fig. 4B, and representative genes in Fig. 4C. The three most frequently melted DNA regions mapped within the first half of the gene and overlapped substantially with the three most AT-rich regions (Fig. 4B). Although the nontranscribed spacer is AT-rich, bubbles were not seen in this region, strengthening the association between topo-bubbles and transcription-induced torsional stress. A gene with two topo-bubbles (black brackets) in AT-rich regions is shown at the top of Fig. 4C, which also shows
We analyzed the position of polymerases flanking topo-bubbles, due to transcription-induced negative torsional stress. AT-rich DNA are consistent with regions of DNA unwinding by topo-bubbles, but the melted region is free of polymerases. Additional genes (with AT-rich regions shaded gray). The nearness of polymerase-free gaps behind elongating polymerases are either blocked upstream or run off downstream of bubbles and also no evidence that neighboring polymerase position is affected by the degree of topological stress (as measured by the degree of DNA melting) and thus no evidence that elongation is affected.

Pileups of closely spaced polymerases are also indicative of slowed elongation, occurring with a certain frequency in WT strains and increasing in elongation mutants (23, 82). Visual inspection of all bubble-containing rRNA genes from the different top1Δ strains indicated that pileups were no more frequent or longer in these genes than in genes from WT cells. Furthermore, the observed pileups were not preferentially upstream of bubbles. This visual inspection was confirmed by quantitative analysis of genes from Pgal7-TOP1 cells after 6 h Top1 depletion (same population analyzed in Fig. 3A and B). That is, pileups of five or more polymerases were not more frequent in bubble-containing genes in Top1-depleted cells (occurring in 22% of genes) than in genes in the control strain not depleted of Top1 (occurring in 37% of genes).

In conclusion, analyses of nearest up- and downstream polymerases as well as polymerase pileups in bubble-containing genes provided no evidence that elongation was slowed by negative torsional stress sufficient to melt the template DNA.

**Topo-bubbles are not seen when elongation rate is slowed in top1Δ cells.** Buble length was similar in different top1Δ strains and in a top1Δ top2-ts strain at permissive temperature (Table 1, Fig. 3A, and Fig. 5A). Based on correlation of the bubble position with AT-richness of the rDNA and the lack of correlation of bubble length with polymerase-free gap size (Fig. 5E), it appears that DNA base composition strongly influences the extent of template melting seen in chromatin spreads. No conditions were noted that influenced bubble length, but three conditions were found that significantly decreased bubble frequency, all of which are predicted to slow Pol I elongation: depletion of Top1 in the absence of RNase H (Fig. 3A) (23), inactivation of Top2 in the absence of Top1 (see below), and treatment of top1Δ cells with MPA, which decreases intracellular guanine nucleotide concentration, thus slowing elongation (6, 67). In the latter experiment, 1-h treatment of SY84 top1Δ cells with MPA resulted in a 8.5-fold decrease in topo-bubbles, from 10.5% of genes with topo-bubbles in untreated cells (n = 95) to 1.6% in treated cells (n = 184). In addition to slowing elongation, these three conditions also increased polymerase density on rRNA genes (23) (Fig. 7 and data not shown). The absence of topo-bubbles may be due to fewer polymerase-free gaps and/or to slowed polymerase movement such that negative torsional stress is less likely to build up. These results support our hypothesis that topo-bubbles represent the buildup of negative torsional stress in polymerase-free gaps behind elongating polymerases.
Single-stranded gene regions exist in vivo in rRNA genes in top1Δ cells. Unconstrained negative topological stress in DNA can be absorbed either by DNA unwinding or by negative supercoiling (Fig. 6A). These inter-convertible configurations have the same linking number (Lk) but differ in twist and writhe. (“Twist” refers to the number of double helical turns [one per 10.5 bp in B-form DNA], and “writhe,” or supercoiling, refers to the number of times the double helix crosses over itself when projected in two dimensions [65].) Since the melted form is favored by conditions encountered in Miller chromatin spreading, i.e., by low ionic strength and by dispersing DNA into a more linear configuration, we sought to determine whether there was evidence that the single-stranded form, rather than the supercoiled form, exists in vivo. We reasoned that if melted DNA exists it is likely to be bound by RPA, which is the most abundant single-stranded DNA-binding protein in eukaryotic cells (34), is present in nucleoli (33), and is expected to quickly bind any ssDNA that forms in the cell (20). This was tested by ChIP analysis using TAP-tagged Rfa1 (TAP-RFA1), the large subunit of the RPA complex, in control and top1Δ strains. EM analysis confirmed topo-bubble occurrence at a typical frequency and length in this top1Δ strain (Table 1). As shown in Fig. 6B, top1Δ cells showed a reproducible increase in Rfa1 bound to transcribed regions of the rDNA compared to control cells, but no increase was seen over the rDNA spacer or the ACT1 gene.

In another approach to determine whether topo-bubbles exist in cells, chromatin spreading was performed following in vivo formaldehyde fixation, as is done in standard ChIP procedures and is generally accepted to capture in vivo interactions (41). Although this fixation rendered chromatin much more difficult to spread, topo-bubbles could be seen in active rRNA genes in top1Δ cells, with two examples shown in Fig. 6C. Thus, based on the increased presence of RPA (Fig. 6B) and the visualization of topo-bubbles after in vivo formaldehyde fixation (Fig. 6C), we conclude that at least some of the torsional stress is absorbed by DNA denaturation in vivo.

When Top2 is inactivated, rRNA genes exhibit signs of positive topological stress. Full-length rRNA is not made if Top1 and Top2 activity are both absent, but there are no major rRNA synthesis defects if either is inactivated individually (11, 23, 66). We visualized rRNA genes after Top2 inactivation to compare their features to those from top1Δ cells. Hundreds of rRNA genes were inspected from top2-ts cells grown for 2 to 3 h at 35°C or for 2 to 4 h at 35°C in the presence of nocodazole to prevent completion of mitosis (29). No topo-bubbles were observed, even in genes with polymerase-free gaps. (A control experiment showed the appearance of topo-bubbles in top1Δ cells at 35°C [data not shown]). Instead, inactivation of Top2 resulted in increased polymerase density on active genes, a finding consistent with slowed elongation, as seen in the nucleolar overviews in Fig. 7A and B comparing genes from permissive (23°C) to Top2-inactivated cells (35°C). Although genes at both temperatures display many nascent transcripts, note the increase in Pol I density after Top2 inactivation as shown by the dense polymerase “backbones” along the rDNA at 35°C (Fig. 7B). This visual impression was confirmed by counting polymerases on >100 active rRNA genes from both permissive and restrictive temperatures (inset Fig. 7A), with results showing a 26% increase in average number of poly-

**FIG. 6.** Evidence that some negative topological stress occurs as melted DNA in top1Δ cells in vivo. (A) Schematic of the two inter-convertible forms of DNA under negative topological stress: melted versus negatively supercoiled. Shown is a hypothetical DNA plasmid of 325 bp, which has a relaxed linking number (Lk0) of 31 (i.e., 325/10.5 = 31). The linking number (Lk) has contributions from twist and writhe, i.e., Lk = twist + writhe. Relaxed plasmid on left has linking number (Lk) of 31 due to 31 helical twists, and thus its ΔLk = 0, where ΔLk = Lk − Lk0 (difference between the actual and the relaxed linking number). On the right is a plasmid of the same length but under negative topological stress (ΔLk = −3), which can be relieved by melting a portion of the DNA or by negative writhe, both of which allow maintenance of stable B-form helix in most of the DNA. (B) ChIP analysis of occupancy of single-stranded DNA-binding protein RPA large subunit (encoded by RFA1) over the rDNA repeat and on ACT1 gene. Shown are the relative ChIP signals in control strain YB169 (TAP-RFA1) versus top1Δ strain YB168 (top1Δ, TAP-RFA1) after normalization to the rDNA5 primer within the rRNA gene. Error bars show standard error of the mean for three independent experiments. (C) EM images of several rRNA genes using modified Miller spreading conditions in which formaldehyde is added to cells prior to cell disruption, mimicking conditions used for ChIP fixation. Arrows indicate topo-bubbles in two nearby genes. Bubbles shown enlarged in the inset. Bar, 0.5 μm.
merases/gene after Top2 inactivation (see the legend for details; $P = 2.6 \times 10^{-9}$).

A second characteristic indicator of slowed elongation was seen in ~15% of rRNA genes as Top2 was inactivated, i.e., a “double gradient” of transcript length, as seen in the gene in Fig. 7D. We previously showed that pre-rRNA cleavage at site A2 to separate small and large subunit pre-rRNAs typically occurs on nascent transcripts at about the time that Pol I reaches the latter third of the gene (37, 56). In WT rRNA genes, this manifests as a series of transcripts in the latter portion of the gene that are shorter than expected and are missing the large 5′-terminal knobs (see Fig. 7C, bracketed transcripts). When elongation was slowed as Top2 was inactivated, A2 cleavage plus subsequent processing events proceeded efficiently, while the polymerases dawdled, resulting in a very obvious double gradient of transcript length (Fig. 7D).
As extrachromosomal rings (36). Although our EM analysis has suggested that Top2 activity are very deficient, many rRNA genes are excised from the template DNA, adding to the evidence that elongation was not slowed by topo-bubbles, as argued above. The simplest explanation for slowed elongation when Top2 is inactivated is increased positive topological stress impeding the forward movement of Pol I.

We next examined rRNA genes in top1Δ top2-ts cells at both permissive and restrictive temperatures (Fig. 7E to H). At permissive temperature (23°C), rRNA genes resembled those from top1Δ cells (Fig. 7E), including many genes with topo-bubbles (e.g., Fig. 7G), as expected due to the absence of Top1. However, at 35°C in the double mutant no bubbles were visible, but a severe processivity defect was observed (Fig. 7F and H). Polymersases and attached transcripts were densely packed toward the 5’ half of the gene and were generally absent at the 3’ end of the gene. That is, rRNA genes had recognizable 5’ ends but were shorter than expected in length (Fig. 7F and H), a finding consistent with the inability of polymersases to elongate to the end of the genes due to extreme positive topological stress in front of the leading polymerase. When both Top1 and Top2 activity are very deficient, many rRNA genes are excised as extrachromosomal rings (36). Although our EM analysis has focused on chromosomal rRNA genes, biochemical evidence indicates a general failure in Pol I processivity in these conditions (23, 66).

These EM results show that inactivation of Top2 results in slowed elongation, a finding consistent with a net increase in positive supercoiling in active rRNA genes. The severe processivity defect seen when both Top1 and Top2 activity are deficient confirms and extends this result seen previously using biochemical methods (23, 66) and demonstrates that results obtained by EM analysis are consistent with results obtained with more standard approaches.

**DISCUSSION**

This report represents the first gene visualization approach to study the in vivo effects of topoisomerase depletion on transcription, focusing on the rRNA gene in its normal chromosomal context, thus bypassing problems that arise when studying genes on circular plasmids (27, 66, 69, 80). The results are summarized in the model in Fig. 8. In comparison to the WT (Fig. 8A), many rRNA genes in cells deficient in Top2 activity (Fig. 8B) exhibited two indicators of slowed elongation rate: higher polymerase density and an advanced (double-gradient) rRNA processing pattern. These results suggest a net

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**FIG. 8.** Model for the effects of Top1 and Top2 depletion on Pol I transcription. (A) In WT cells, Top1 and Top2 maintain the rDNA template in a relatively relaxed topological form, as shown by the relaxed DNA between polymersases. WT rRNA genes exhibit a characteristic rRNA processing pattern, consisting of compaction of pre-small subunit RNA into a large 5’-terminal knob (SSU processome), followed by cotranscriptional cleavage to separate small subunit pre-rRNA (released and no longer visible in chromatin spreads) from large subunit pre-RNA (still extruding from the polymerase) (37, 56). (B) In cells deficient in Top2 activity, polymersases are tightly packed on most rRNA genes, and cotranscriptional cleavage is often “advanced” (double-gradient pattern) due to continued processing on dawdling polymersases. Both of these features are characteristic of slow elongation, which is consistent with increased positive torsion (“+” signs) and/or supercoiling in the absence of Top2. Although DNA writhing is not seen in EM spreads, its in vivo presence is inferred (as shown by the positive supercoil) since writhed DNA is the substrate for Top2 (61). (C) In cells deficient for both Top1 and Top2 activity, polymersases are rarely seen to transcribe beyond the first half of the gene, which is consistent with DNA so tightly wound (“+” signs and positive writhe) that it resists the unwinding necessary for transcription (23, 66). (D) In Top1-deficient cells, many genes show no evidence of topological abnormalities, presumably due to the action of Top2 and to local dissipation of transcription-induced positive and negative stress between elongating polymersases (“+” and “−” signs between polymerases). Excess negative torsion (due to more widely spaced polymersases) results in DNA breathing which favors the formation of R-loops (the top gene in panel D). Such R-loops are transient due to endogenous RNase H activity (see reference 23 and results in the present study). In many genes (e.g., the bottom gene in panel D), sufficient negative torsion accumulates to melt the template DNA, particularly in AT-rich regions. Evidence suggests that the melted DNA is bound by RPA (peanut shapes on bubble). (E) When RNase H activity is compromised in Top1-deficient cells (P<sup>top1</sup>-top2-ts rnh201Δ), topo-bubble formation greatly decreases (Fig. 3), and transcript density increases (23). We propose that undigested R-loops significantly slow Pol I elongation, disallowing the accumulation of hypernegative topological stress and the formation of topo-bubbles.

This double-gradient pattern, which is also sometimes seen after depletion of Top1 in rnhΔ strains (Fig. 3B, third gene down), was not seen on bubble-containing genes from top1Δ strains, adding to the evidence that elongation was not slowed by topo-bubbles, as argued above. The simplest explanation for slowed elongation when Top2 is inactivated is increased positive topological stress impeding the forward movement of Pol I.
increase in positive torsion in active genes in cells lacking Top2 activity (Fig. 8B). In cells deficient for both Top1 and Top2 activity (Fig. 8C), polymerases were rarely seen to progress beyond the first half of the gene, which is consistent with DNA so tightly wound as to resist the unwinding necessary for transcription (23, 66). Although DNA writhing is not seen in our chromatin spreads, its in vivo presence is inferred when Top2 activity is deficient (Fig. 8B and C) since wrinkled DNA is the Top2 substrate (61, 65).

In cells deficient for Top1 (Fig. 8D) many active genes accumulated high levels of negative torsional stress, which was dependent on RNase H activity (top gene) and appeared as melted DNA (bottom gene). The combined absence of Top1 and RNase H (Fig. 8E) greatly decreased topo-bubble formation (Fig. 3A) while increasing Pol I pausing and DNA:RNA hybrid occupancy on the gene (23). These results indicate a role for RNase H in accumulation of hypernegative topological stress, which we attribute to its role in digesting R-loops, allowing continued transcription. Although we did not see any convincing R-loops in the presence or absence of RNase H (perhaps because they may be considerably shorter than topo-bubbles and/or less amenable to visualization by our EM approach), there is suggestive evidence to include them in our model. Thus, a transient R-loop is shown in a region where DNA is beginning to unwind (Fig. 8D), and undigested R-loops are shown impeding transcription when both Top1 and RNase H are deficient (Fig. 8E).

**Does negative topological stress exist as melted DNA in vivo?** In highly transcribed genes, the negative torsion generated behind one polymerase may be cancelled by the positive torsion generated ahead of the next one, lessening the need for topoisomerase activity (Fig. 8A and D) (46, 76). However, this canceling effect is lost if a polymerase is not followed closely by another polymerase, allowing negative torsional stress to accumulate in the resulting polymerase-free gap. Such stress can be absorbed either by partial DNA melting or by increased negative writhe (Fig. 6A), both of which allow most of the DNA to maintain a relatively constant twist near 10.5 bp/turn, which is preferred due to the stability conferred by base-pairing and stacking (8, 60, 65). Our observations of DNA bubbles in active genes after in vivo cross-linking (Fig. 6C), together with the increased presence of the ssDNA binding protein RPA in the gene (Fig. 6B), suggest that DNA melting absorbs at least some of the negative stress in cells. Additional indirect evidence for the existence of topo-bubbles is that they are not relaxed by Top2, which would be able to resolve the crossovers in negatively wrinkled DNA, but would not recognize underwound DNA. The greatly enhanced psoralen accessibility of rDNA observed in top1Δ cells but not in Top2-deficient cells (15) also points to a loosened rather than a supercoiled structure in the former but not the latter, in agreement with our results.

Dynamic breathing in torsionally stressed DNA, which is more likely to occur in AT-rich regions, would provide an opportunity for RPA to bind. Such binding is energetically favorable because it relaxes negative torsional stress and is self-limiting, proceeding until the driving stress is removed (20, 54). RPA needs only a short (8- to 10-nt) region of ssDNA to initiate binding and in vitro studies have shown RPA-induced unwinding at AT-rich regions on plasmids under negative torsional stress (73), similar to the scenario we propose here.

**Relationship of topo-bubbles to RNase H activity and R-loops.** One explanation for the dependency of topo-bubble formation on RNase H activity is that elongation is slowed in its absence (due to unresolved R-loops) such that sufficient negative stress does not accumulate (Fig. 8E). It is also possible that transient R-loop formation plays a more direct role in DNA melting, e.g., the strand separation that occurs in R-loop formation might provide the entry point for RPA binding and topo-bubble formation rather than, or in addition to, dynamic breathing in the DNA as proposed above.

Extensive studies in E. coli have examined the interplay between topoisomerase activity, negative torsion, and R-loop formation (21). Although results in E. coli and yeast are not directly comparable due to differences in topoisomerase specialization (such as no gyrase counterpart in eukaryotes) and differences in the ability of endogenous RNase H to efficiently digest R-loops (2, 31), the results agree that transcription-induced negative torsion leads to R-loop formation, which contributes to elongation pauses and aberrant cleavage of nascent RNA (3, 23). It appears that S. cerevisiae is more efficient than E. coli in digesting R-loops (consistent with the more highly processive nature of eukaryotic RNase H [16]), thus allowing continued transcription and viability in top1Δ strains but leading to significant negative torsional stress in active genes.

**Quantifying negative superhelical density.** In previous studies, transcription-induced negative torsional stress has been found to dissipate upstream of active promoters and may play a role in activating transcription or replication at a nearby origin (42, 72, 80). Although we cannot rule out that this happens for a short distance upstream of the rDNA promoter or that it happens but does not melt the template, we found that topo-bubbles were not seen to extend beyond the limits of the transcribed gene even though the nontranscribed spacer is quite AT-rich (Fig. 4B). This finding is consistent with the lack of correlation between active transcription of rRNA genes and firing of the adjacent upstream replication origin (52). DNA-binding proteins and chromatin structures can form barriers that impede diffusion of torsional stress and thus increase localized supercoiling (43, 64). The promoter-bound UAF complex, which includes histones H3 and H4 and is essential for Pol I transcription and rDNA silencing, is a strong candidate for blocking such dissipation (55).

In estimating superhelical density in affected rRNA genes, we used the 6,740-bp gene as the unit in which stress can dissipate for reasons just discussed. This length of DNA will have a relaxed linking number (Lk0) of 642 (6,740/10.5). Assuming 41 twists of the DNA helix, we used the 6,740-bp/h/11002 gene as the unit in which stress can dissipate for reasons just discussed. This length of DNA will have a relaxed linking number (Lk0) of 642 (6,740/10.5). An inactive rRNA gene will have constrained negative supercoiling due to nucleosomal packaging, but the linking number will decrease upon activation and loss of nucleosomes (51), which constrain ~1 negative supercoil each (59). Assuming ~41 nucleosomes per gene (6,740 bp/165 bp/nucleosome = ~41), this corresponds to a ΔLk of ~41 and a superhelical density (σ, where σ = ΔLk/Lk0) of ~0.06 for a nucleosome-free rRNA gene in control strains. The average topo-bubble was 415 bp long, corresponding to a loss of ~40 twists of the DNA helix. Thus, ΔLk increases to ~81 (~41 + ~40) and σ = ~0.13 in the average bubble-containing gene from top1Δ cells, about
twice the normal value. By similar calculations, genes with higher levels of melting (up to 2 kb or 30% of the gene length) due to either single large bubbles or, more frequently, to multiple smaller bubbles, have $\sigma = -0.37$, or 6 times the normal value. For comparison, the AT-rich FUSE element in the human MYC promoter melts when $\sigma$ reaches $-0.07$ (40). Transcription-induced negative superhelical density in top1\(\Delta\) cells has been reported to be more than twice the normal value (12), with more precise determination limited by the chloroquine gel method used to count topoisomers.

**Transcription in genes with high negative torsion.** Although unconstrained negative supercoiling has been detected in the rDNA of normal cells (47), it was somewhat surprising that genes with such high levels of negative $\sigma$ continued transcribing and that top1\(\Delta\) cells grew quite well even when 30% of the rRNA genes displayed template melting. For example, hypernegative supercoiling causes growth inhibition in *E. coli* (21), and although transcription initiation increases as $-\sigma$ increases, this is true only to a certain point ($\sigma \sim -0.07$), after which it decreases (58). We presented evidence that transcription elongation was not slowed in genes with topo-bubbles. We also observed that polymerases were not seen within topo-bubbles. We also showed that polymerases were not seen within topo-bubbles, indicating that Pol I does not transcribe the single-stranded template. Thus, hypernegative topological stress (either as negatively writhed or melted DNA) forms no barrier to polymerase elongation, indicating that any denatured DNA reanneals as it enters the polymerase. This helical reformation would be driven by the powerful force of multiple RNAPs (4, 24) and by transcription-induced positive torsion in DNA entering the polymerase, which would cancel the negative torsion and facilitate reannealing and removal of RPA (20). Thus, as transcription continues, the melted DNA might be transiently reannealed—only to reappear as the polymerase moves down the gene. The preferential occurrence of bubbles in AT-rich regions supports their transient existence, and their dynamic appearance probably requires contributions from polymerase positions, negative torsion, and base composition of the template. In molecular simulations, denaturation bubbles due to negative torsional stress are very dynamic, moving erratically along DNA and converting to other topologies (60, 74).

**Functions of Top1 and Top2 during Pol I transcription.** Both Top1 and Top2 can relax positive and negative supercoiling and are abundant in the nucleolus. The fact that their absence results in the inability of polymerases to anneal—only to reappear as the polymerase moves down the gene supports their transient existence, and their dynamic appearance probably requires contributions from polymerase positions, negative torsion, and base composition of the template. In molecular simulations, denaturation bubbles due to negative torsional stress are very dynamic, moving erratically along DNA and converting to other topologies (60, 74).

**ACKNOWLEDGMENTS**

This study was supported by Public Health Service grants GM63952 (A.L.B.) and GM04444 (M.M.S.) from the NIGMS, by American Heart Association grant 0755633U (J.S.S.), and by the Wellcome Trust (D.T.). We thank Dan Burke and Rodney Rothstein for yeast strains.

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