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Positive supercoiling of DNA greatly diminishes mRNA synthesis in yeast

(DNA topoisomerases/DNA topology/transcription)

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ABSTRACT **In *Saccharomyces cerevisiae* cells harboring a *GAL1* promoter-linked β-galactosidase gene, the simultaneous expression of *Escherichia coli* DNA topoisomerase I and inactivation of yeast DNA topoisomerases I and II reduces the cellular level of β-galactosidase to an undetectable level. Analysis of intracellular mRNA level and the density of RNA polymerase along DNA indicates that this reduction is due to the suppression of transcription and that both plasmid-borne and chromosomally located genes are affected. These results are interpreted in terms of inhibition of transcription *in vivo* due to positive supercoiling of the DNA template: preferential removal of transcription-generated negative supercoils by *E. coli* DNA topoisomerase I in the absence of both yeast DNA topoisomerases I and II results in the accumulation of positive supercoils in intracellular DNA. In normal prokaryotic or eukaryotic cells, accumulation of positive supercoils is presumably avoided through the balanced actions of DNA topoisomerases.**

In prokaryotes, there is substantial evidence that gene expression is often influenced by the cellular levels of DNA topoisomerases and, by implication, the state of supercoiling of intracellular DNA (1–3). Whether the same is true in eukaryotes is less clear. There is evidence that in eukaryotic as well as prokaryotic cells, transcription may lead to supercoiling of the template DNA, and one of the functions of DNA topoisomerases is the removal of supercoils (4, 5). Eukaryotic DNA topoisomerase I is known to be preferentially associated with actively transcribed regions of chromatin, and this association is generally assumed to reflect a functional role of the enzyme in transcription (2, 6–8). Yeast mutants devoid of the enzyme exhibit no abnormality in transcription, however (9). In these *top1* mutants, the normal roles of DNA topoisomerase I might be fulfilled by DNA topoisomerase II, as both enzymes are capable of relaxing positively and negatively supercoiled DNA *in vivo* (5, 10). Simultaneous inactivation of DNA topoisomerases I and II, through the use of *top1 top2* double-mutants, reduces rRNA synthesis by a factor of 10 and total poly(A)$^+$ RNA synthesis by a factor of 3 (11, 12). These reductions can be attributed to changes in the topology of the DNA template, but a direct link is difficult to establish because of the multiple cellular roles of the topoisomerases. A causal relation between template topology and proficiency is further clouded by the finding that transcription of several specific *Saccharomyces cerevisiae* genes appears to be unaffected by inactivation of both DNA topoisomerases I and II (13).

Expression of *Escherichia coli* topoisomerase I in the absence of active yeast topoisomerases I and II leads to positive supercoiling of intracellular DNA (5). This phenomenon has been interpreted in terms of the twin-domain model of transcriptional supercoiling (14): a transcriptional ensemble moving along its template generates positive supercoils ahead of it and negative supercoils behind it if there is sufficient resistance against the circling of the macromolecular complex around the DNA; positive supercoils accumulate when the negative supercoil-specific bacterial topoisomerase becomes the only intracellular relaxation activity. In this communication, we show that accumulation of sufficient positive supercoils in template DNA greatly reduces mRNA synthesis in yeast, probably at the initiation step.

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MATERIALS AND METHODS

Yeast Strains and Plasmids. Strains CH1106 (α *ade2-101 Δleu2 lys2-801 Δtrp1 ura3-52 top2-4*) and its *TOP2*$^+$ sibling CH1105 were kindly provided by Connie Holm, Harvard University. JCW7 (α *ade2-101 Δleu2 lys2-801 Δtrp1 ura3-52 Δtop1::URA3*) and JCW8 (α *ade2-101 Δleu2 lys2-801 Δtrp1 ura3-52 Δtop1::URA3 top2-4*) were derived from CH1105 and CH1106, respectively (Raymond A. Kim and J.C.W., unpublished results), by the one-step gene-transplacement procedure (15). Targeted deletion of the inserted *URA3* marker in these strains gave the corresponding *ura3* derivatives, JCW9 and JCW10.

All plasmids contained the cis-acting sequences of the yeast 2-μm plasmid that are necessary for stable propagation in yeast. pRY131 contains a *GAL1::lacZ* fusion and *URA3* marker (16). YEp*topA*-PGPD (5) contains the *E. coli topA* gene downstream of the promoter of the yeast *GPD* gene (encoding glyceraldehyde-3-phosphate dehydrogenase), as well as a *TRP1* marker. YEpY1 contains the wild-type yeast DNA topoisomerase II gene, with its own promoter, and a *URA3* marker. This plasmid was originally isolated from a yeast genomic library constructed by inserting fragments of a partial *Sau*3A1 restriction endonuclease digest of yeast DNA into the *Bam*HI site of YEp24; a restriction map of the entire 10-kilobase (kb) insert, and the nucleotide sequence of half of the insert, containing the *TOP2* gene, have been reported (17). YEp*LYS2* is a YEp24 derivative with the *URA3* gene replaced by *LYS2* and the region between the *Aat* II and *Xba I* restriction endonuclease sites of YEp24 deleted. In this plasmid, *LYS2* transcription proceeds in the same direction as transcription of the β-lactamase gene.

β-Galactosidase Assays. Assays for β-galactosidase were performed according to Yocum *et al.* (16). Pairs of cultures containing cells harboring pRY131 or both pRY131 and YEp*topA*-PGPD were grown to logarithmic phase in an appropriate synthetic drop-out medium containing 2% (wt/vol) raffinose. At time 0, aliquots of the samples were taken for β-galactosidase assays and each of the remaining cultures was split into two equal portions for continued incubation at 26°C and 35°C, respectively. One hour afterwards, galactose was added (2%, wt/vol) to the cultures to induce β-galactosidase synthesis from the *GAL1* promoter; 5 hr later, cells from all cultures were harvested for measurements of β-galactosidase activity.

Determination of Intracellular mRNA Levels by Primer Extension. Cultures were grown to logarithmic phase in the appropriate synthetic drop-out medium, usually containing 2% raffinose as the carbon source, before shifting from growth at 26°C to 35°C, the latter being a nonpermissive temperature for strains carrying the *top2-4* mutant DNA topoisomerase II gene. Total nucleic acid was isolated from $\approx 10^8$ cells disrupted by grinding with glass beads (18). Each sample was resuspended in ≈20 μl of water; the precise resuspension volumes for different samples were adjusted according to the final cell densities of the cultures, so as to give lysates of the same cell concentrations. A mixture of *URA3*- and *ADH1*-specific primers was added to 5 μl of each lysate, and primer extension was carried out as described (19). The transcription start sites of both genes have been identified previously (20, 21). The primers used here were 5′-GCGGATAATGCCTTTAGCGGC-3′ and 5′-CGTAGAAGATAACACCTTTTTG-3′ for *URA3* and *ADH1*, respectively. Either plasmid pRY131 or plasmid YEpY1 provided the *URA3* template monitored in these experiments; the chromosomal *ura3-52* allele in the strains has a Ty insertion upstream of the primer target (22). All experiments were performed in parallel with cells cotransformed with YEp*topA*-PGPD. Primer-extension products were separated by electrophoresis in a denaturing 5.5% polyacrylamide gel and detected by autoradiography.

Run-On Transcription Analysis of a Plasmid-Borne *LYS2* Gene. JCW10 (Δ*top1 top2-4*) was transformed with either YEp*LYS2* alone or YEp*LYS2* and YEp*topA*-PGPD together. Approximately 10^8 cells were permeabilized and run-on transcripts were generated according to published procedures (23). Total nucleic acid was isolated as described above and was prepared for hybridization according to Greenberg and Ziff (24). Five DNA fragments from evenly spaced regions of *LYS2* coding sequences, each 450–600 base pairs in length, were used to probe the run-on transcripts. Ten micrograms of each linearized, denatured probe was fixed to a GeneScreen membrane (DuPont) by UV irradiation. Hybridization and washing conditions were those of Church and Gilbert (25).

Two-Dimensional Agarose Gel Electrophoresis for Analysis of the Linking-Number Distributions of Plasmids. Total nucleic acid was isolated as described in the section on primer extension. To 5 μl of each sample, 10 μg of RNase A was added and the mixture was incubated at 37°C for 15 min and then loaded directly in one of the four equally spaced sample wells of a 0.7% agarose gel (20 cm × 20 cm × 0.5 cm) containing TBE (0.1 M Tris borate/2 mM EDTA, pH 8.3) plus 0.6 μg of chloroquine per ml. After electrophoresis in the first dimension (top to bottom), the gel slab was equilibrated with TBE plus 3 μg of chloroquine per ml and electrophoresis in the second dimension (left to right) was then performed in the same buffer. DNA was transferred to a GeneScreen membrane (DuPont) and probed with radiolabeled *LYS2* or *URA3* DNA prepared with a random-priming kit (Stratagene). Hybridization and washing conditions were the same as above.

RESULTS

β-Galactosidase Activity Is Greatly Reduced Under Conditions Leading to Positive Supercoiling of Intracellular DNA. An isogenic set of yeast DNA topoisomerase mutants harboring pRY131 (16), in which the expression of *E. coli* β-galactosidase is from a galactose-inducible yeast *GAL1* gene promoter, was used to examine the effects of template topology on β-galactosidase level. JCW10 (Δ*top1 top2-4*) cells grown at 35°C showed a moderate, 4-fold reduction in β-galactosidase level relative to cells grown at 26°C (Table 1). This reduction is likely to be due partly to the cessation of growth of the double mutant at the nonpermissive temperature; the 5-hr induction period corresponds approximately to one doubling time for this strain at 26°C. Thus, in agreement with published results (12, 14), no major difference in the expression of β-galactosidase from the galactose-inducible GAL1 promoter was observed when both topoisomerases were inactivated.

Table 1. Variation of β-galactosidase level with topoisomerase activity in yeast topoisomerase mutants

Strain	Temp., °C	β-Galactosidase
JCW10 (Δ*top1 top2-4*)[pRY131]	26	200
	35	50
JCW10(Δ*top1 top2-4*)[pRY131, YEp*topA*]	26	1050
	35	<1
CH1106 (*TOP1*$^+$ *top2-4*)[pRY131]	26	450
	35	120
CH1106 (*TOP1*$^+$ *top2-4*)[pRY131, YEp*topA*]	26	1090
	35	130
JCW9 (Δ*top1 TOP2*$^+$)[pRY131]	26	450
	35	460
JCW9 (Δ*top1 TOP2*$^+$)[pRY131, YEp*topA*]	26	280
	35	280

The level of β-galactosidase in each sample is relative to that of the same sample immediately before its splitting for incubation at 26°C and 35°C. The levels in the various reference samples were within a factor of 4.

The same experiment gave a strikingly different result, however, in JCW10 (Δ*top1 top2-4*) cells harboring an additional plasmid YEp*topA*-PGPD (5), in which *E. coli* DNA topoisomerase I is expressed constitutively from a yeast promoter. Whereas the culture grown at the permissive temperature showed a high level of β-galactosidase, no activity was detectable in the culture grown at the nonpermissive temperature.

A priori, the preferential removal of negative supercoils by the bacterial enzyme (26) could by itself abolish transcription in yeast. Earlier studies implicating a link between transcription and negative supercoiling of the DNA template in eukaryotes have been reviewed (2, 27). More recently, it has been reported that transcription decreases the linking numbers of eukaryotic plasmids. In yeast, for example, transcription appears to slightly reduce plasmid linking number in Top$^+$ strains (28), and hypernegatively supercoiled plasmids were found in *top1* and *top1 top2* mutants when the plasmids were heavily transcribed (13). The paradoxical stimulation of transcription of plasmid-borne rRNA genes and inhibition of transcription of chromosomal rRNA genes upon inactivation of both yeast DNA topoisomerases I and II were attributed to the negative supercoiling of the template in the absence of the topoisomerases (8). It has also been shown that eukaryotic DNA topoisomerase II, in combination with a protein factor purified from the silk gland of *Bombyx mori*, can negatively supercoil DNA and stimulate transcription in an *in vitro* eukaryotic RNA polymerase II transcription system (29).

Data shown in Table 1 for the other strains, however, suggest that removal of negative supercoils by the bacterial enzyme is unlikely to be the direct cause of abolishing transcription; rather, the precipitous drop in β-galactosidase in yeast cells lacking functional yeast DNA topoisomerases I and II and expressing *E. coli* DNA topoisomerase I is most likely due to the accumulation of positive supercoils in intracellular DNA under these conditions (5). Neither the inactivation of yeast DNA topoisomerase I, II, or both simultaneously nor the expression of *E. coli* DNA topoisomerase I in the presence of a functional yeast DNA topoisomerase I or II can abolish the expression of β-galactosidase;

only the combination of inactivating both yeast topoisomerases and expressing the *E. coli* enzyme leads to inhibition.

Positive Supercoiling Suppresses Expression of Plasmid-Borne and Chromosomal Genes at the Transcriptional Level. Results of two types of experiments support the notion that positive supercoiling of intracellular DNA suppresses expression of β-galactosidase from the yeast *GAL1* promoter, as well as gene expression from other yeast promoters, at the transcriptional level. In the first, primer-extension analysis was used to quantitate mRNA expressed from a plasmid-borne *URA3* gene and the chromosomally located *ADH1* gene. In JCW10 (Δ*top1 top2-4*) expressing *E. coli* DNA topoisomerase I, both *URA3* and *ADH1* mRNA levels dropped steeply when the temperature was shifted to 35°C; in the same strain devoid of *E. coli* DNA topoisomerase I, changes in these mRNA levels were much less pronounced following the temperature shift (Fig. 1*A*). Synthesis of *lacZ* mRNA from the *GAL1* promoter behaved similarly (data not shown). Analysis of the linking-number distribution of the plasmid containing the *URA3* gene, by two-dimensional gel electrophoresis in the presence of different amounts of chloroquine during the first- and second-dimension electrophoresis (30), showed that the time course of positive supercoil accumulation in the *URA3* plasmid coincided with that of mRNA reduction (results not shown; see Fig. 2 for an analogous experiment). As the decrease in the transcription of the chromosomal *ADH1* followed a similar time course as that of the plasmid-borne *URA3*, it can be inferred that accumulation of positive supercoils in chromosomal DNA is not grossly different from that in plasmids. Transformation of JCW10 to *TOP2*$^+$ with a plasmid, YEpY1, containing the entire *TOP2* gene abolished the dependence of the *URA3* mRNA level on *E. coli* DNA topoisomerase I at 35°C (Fig. 1*B*). Control experiments using strains with wild-type *TOP1* or *TOP2*—namely, CH1106 (*TOP1*$^+$ *top2-4*) or JCW9 (Δ*top1* *TOP2*$^+$)—also showed that the mRNA levels of both the plasmid-borne *URA3* and chromosomal *ADH1* in these strains were not severely affected by shifting the temperature from 26°C to 35°C, whether the cells were expressing *E. coli* DNA topoisomerase I or not (data not shown).

In a second series of experiments, run-on transcription assays (23) were carried out to estimate the density of functional RNA polymerase molecules along a *LYS2* gene on a multicopy plasmid, YEp*LYS2*. Five DNA fragments from evenly spaced regions of the *LYS2* coding sequences were used to probe the run-on transcripts. Data obtained with a 460-base probe complementary to the first 1.1–1.6 kilobases of *LYS2* mRNA are depicted in Fig. 2*A*. In JCW10 (Δ*top1*

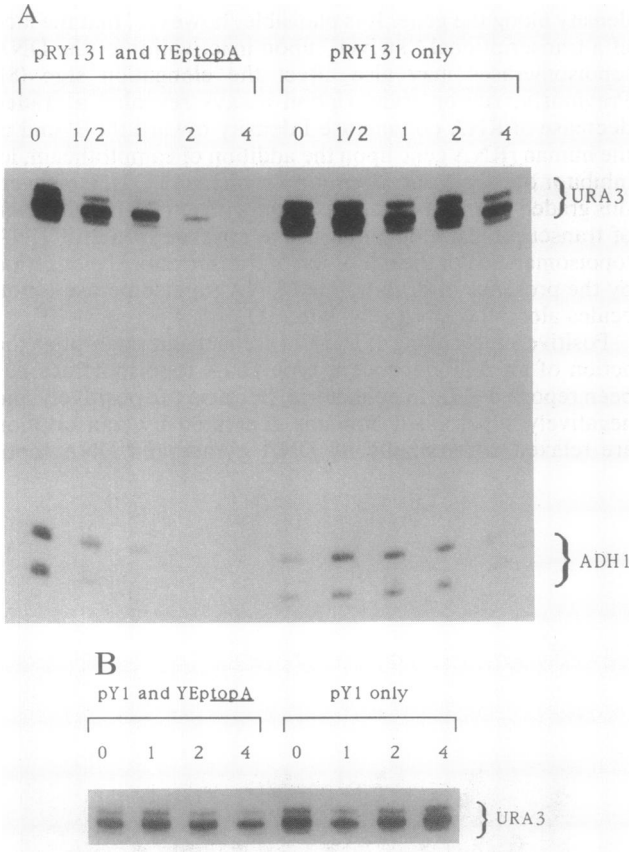

FIG. 1. Intracellular mRNA levels in the double yeast DNA topoisomerase mutant JCW10 (Δ*top1 top2-4*). (*A*) Levels of plasmid pRY131-encoded *URA3* mRNA and chromosomal *ADH1* mRNA were quantitated by primer extension; the number above each lane gives the time in hours following a shift of the growth temperature to 35°C to inactivate DNA topoisomerase II. Samples shown in the left half were from cells which also harbored an *E. coli* topoisomerase I (*topA*) expression plasmid, YEp*topA*-PGPD (5); samples in the right half were from cells without the *topA* plasmid. Cultures were grown in 2% raffinose; substitution of glucose or galactose for raffinose did not change the results significantly. (*B*) Levels of *URA3* mRNA from a *URA3* gene on YEpY1, a 2-μm plasmid derivative which also carries the wild-type yeast *TOP2* gene including its promoter. Otherwise, experimental design was identical to that described in *A*.

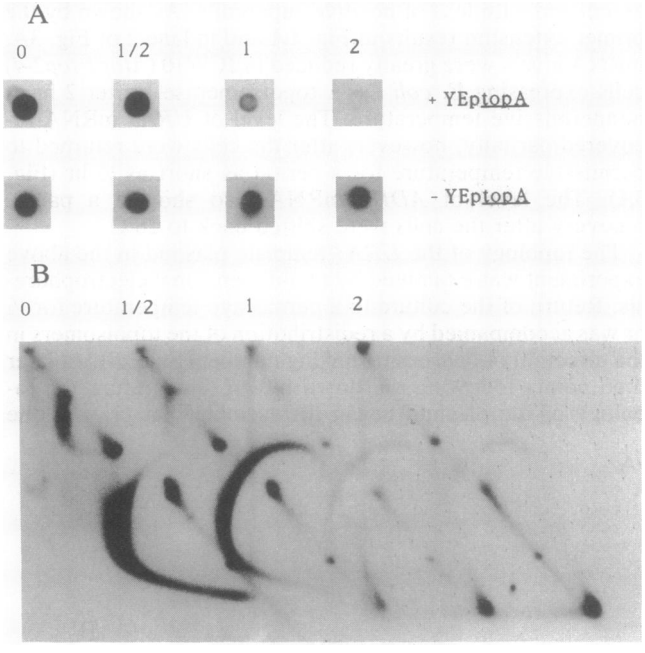

FIG. 2. Run-on transcription analysis of a plasmid-borne *LYS2* gene. (*A*) Run-on transcription analysis of a *LYS2* gene on YEp*LYS2* in JCW10 (Δ*top1 top2-4*); the number above each column of dots denotes the time in hours after shifting the growth temperature to 35°C. Samples shown in the top row were from cells which also harbored YEp*topA*-PGPD; samples in the bottom row were from cells in which YEp*topA*-PGPD was absent. In the blots shown, a radiolabeled 460-bp *Spe* I–*Nco* I DNA fragment complementary to the first 1.1–1.6 kilobases of *LYS2* mRNA was used as the probe. (*B*) Two-dimensional agarose gel analysis of the linking-number distributions of the *LYS2* plasmid used in the run-on transcription experiments. The two-dimensional electrophoresis technique separates covalently closed DNA topoisomers of different linking numbers into an arc; topoisomers of progressively higher linking numbers align clockwise along this arc (30). When the DNA becomes highly positively supercoiled, the topoisomers coalesce into a spot slightly above the diagonal under the gel electrophoresis conditions employed. Four samples were analyzed in this gel. The upper tip of each diagonal line corresponds to the position of each sample-well, and the number above it specifies the time in hours following the shift in growth temperature.

top2-4) cells expressing *E. coli* DNA topoisomerase I, the amount of run-on transcripts hybridizable to this probe dropped steadily upon shifting of the temperature to 35°C; such a drop was not observed in JCW10 cells without the *E. coli* enzyme. Use of the other four fragments as probes yielded essentially the same hybridization patterns as those shown in Fig. 2*A*. These results indicate that when *E. coli* topoisomerase I is expressed in the absence of yeast DNA topoisomerases I and II, there is a uniform reduction of the density of active RNA polymerase molecules along the *LYS2* gene.

Fig. 2*B* depicts the topological state of the plasmid that served as the template in the run-on experiments described above. In the double yeast DNA topoisomerase mutant expressing *E. coli* DNA topoisomerase I, the appearance of positively supercoiled YEp*LYS2* and the decrease in *LYS2*-bound RNA polymerase molecules follow a similar time course after shifting of the cells to nonpermissive temperature (compare Fig. 2*B* with the top half of Fig. 2*A*). Highly positively supercoiled DNA is noticeable in the ½-hr sample and is very prominent in the 1- and 2-hr samples.

Changes in Transcriptional Level and Template Topology upon Return of Yeast Δ*top1 top2-4* Cells to Permissive Temperature. Further evidence of direct relationship between transcriptional inhibition and positive supercoiling is the demonstration that recovery from transcriptional inhibition is associated with loss of positive supercoils. As shown by the primer extension results in Fig. 1*A* and in lane 2 of Fig. 3*A*, mRNA levels were greatly reduced in JCW10 (Δ*top1 top2-4*) cells expressing *E. coli* DNA topoisomerase I after 2 hr at nonpermissive temperature. The level of *URA3* mRNA recovered partially, however, after the cells were returned to permissive temperature for a period as short as ½ hr (Fig. 3*A*). The level of *ADH1* mRNA also showed a partial recovery after the cells were shifted back to 26°C.

The topology of the *URA3* template plasmid in the above experiment was examined by two-dimensional electrophoresis. Return of the culture to a permissive temperature for ½ hr was accompanied by a redistribution of the topoisomers in the direction of decreasing linking number (Fig. 3*B*). At later time points following the downshift in temperature the topology of the plasmid began to resemble that prior to the upshift in temperature. These results provide further evidence that transcriptional inhibition correlates with positive supercoiling of the DNA template.

DISCUSSION

Previously it was reported that transcription under certain conditions could alter the topology of intracellular DNA (3–5, 13, 28). The data presented here show that changes in DNA topology, in turn, can have a profound effect on transcription in a eukaryote. In yeast cells lacking active yeast DNA topoisomerases I and II, the presence of *E. coli* DNA topoisomerase I permits the removal of negative supercoils only. Because transcription generates both negative and positive supercoils in the DNA template (14), preferential removal of negative supercoils leads to the accumulation of positive supercoils. As the DNA template becomes more positively supercoiled, the level of transcription progressively decreases. Presumably, a steady-state level of positive supercoiling of the template is reached asymptotically at which there is little transcription.

The run-on transcription data are consistent with the notion that as positive supercoils accumulate in the *LYS2* DNA template, initiation of transcription of the gene is reduced, leading to a uniform decrease in RNA polymerase density along the gene. It is plausible, however, that the lack of a transcriptional "swivel" upon inactivation of the DNA topoisomerases may also affect the elongation step (8). Previously, run-on transcription assays revealed a graded decrease of RNA polymerase I density toward the 3′ end of the human rRNA gene upon the addition of camptothecin, an inhibitor of eukaryotic DNA topoisomerase I (31). However, this graded distribution is consistent with either the inhibition of transcriptional elongation in the absence of active DNA topoisomerase I or the inhibition of transcriptional elongation by the presence of drug-trapped DNA topoisomerase I molecules along the DNA template (31).

Positive supercoiling of DNA in archaebacteria through the action of an ATP-dependent type DNA topoisomerase has been reported (32). In eubacteria, because the positively and negatively supercoiled domains generated by transcription are relaxed differentially by DNA gyrase and DNA topo-

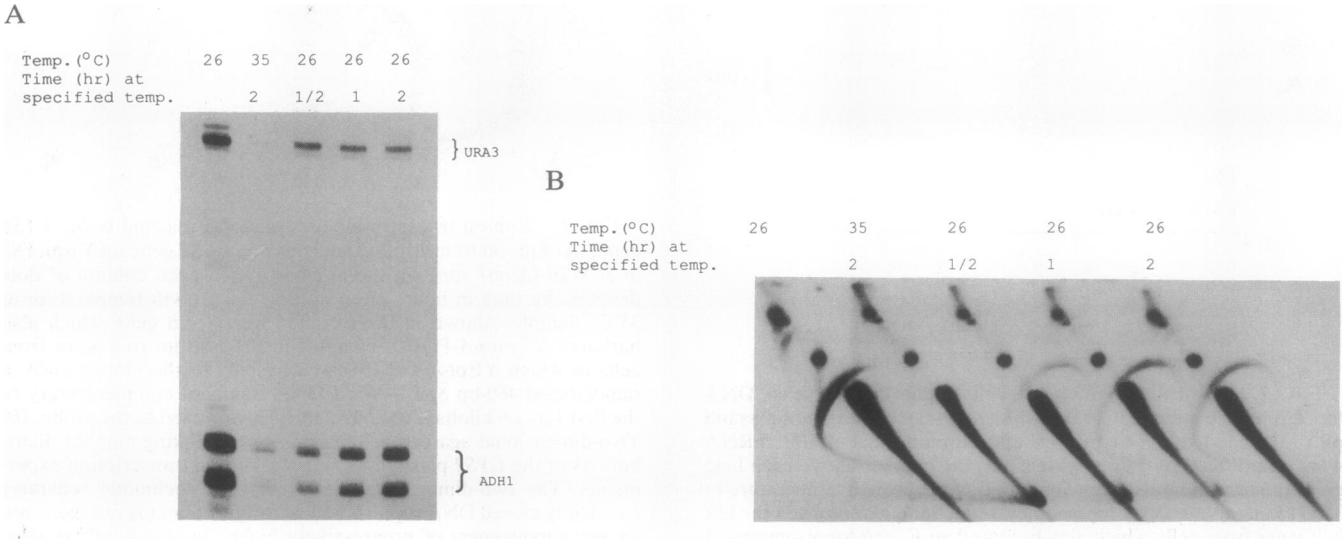

FIG. 3. Analysis of mRNA levels and the distribution in the linking numbers of a plasmid DNA after return of JCW10 (Δ*top1 top2-4*) cells from a nonpermissive temperature of 35°C to a permissive temperature of 26°C for DNA topoisomerase II. Cells were first grown at 26°C, shifted to 35°C for 2 hr, and then returned to 26°C. (*A*) Levels of plasmid pRY131-encoded *URA3* mRNA and chromosomal *ADH1* mRNA were quantitated by primer extension. From left to right, the lanes represent successive time points in the experiment, with the time elapsed at the specified growth temperatures indicated above each lane. The leftmost lane corresponds to the initial conditions immediately preceding the shift to 35°C. (*B*) Analysis of the linking-number distributions of pRY131 by two-dimensional gel electrophoresis. The same samples used in the analysis shown in *A* were used here.

isomerase I, inhibition of gyrase leads to the accumulation of positive supercoils (4, 14), which may in turn interfere with transcription. Interestingly, for several genes inhibition of DNA gyrase in a *topA*$^+$ genetic background actually increases the level of transcription (2, 3). Whether the DNA templates become positively supercoiled under the conditions employed in the latter experiments is uncertain; these findings nevertheless suggest that in eubacteria, positive supercoiling may not repress the transcription of all genes.

In eukaryotes, the enrichment of DNA topoisomerase I in actively transcribed regions presumably prevents excessive supercoiling of the template. Even inactivation of both yeast DNA topoisomerases I and II does not generally lead to the accumulation of positive supercoils, due to the absence of an endogenous activity that can remove negative supercoils efficiently and specifically (5). Recent studies of yeast DNA topoisomerase III suggest, however, that this enzyme may contribute significantly to the preferential removal of negative supercoils in certain regions of intracellular DNA (33).

Finally, although accumulation of positive supercoils over a long period of time is probably rare under physiological conditions in eukaryotes with wild-type DNA topoisomerases, positive as well as negative supercoils are probably present transiently at all times. How such waves might affect physiological processes is of interest. It has been suggested, for example, that waves of supercoils might affect nucleosome structure in a DNA template undergoing transcription (34–37). The plausible effects of the supercoil waves of one transcript on gene expression and other cellular processes nearby are also intriguing, and whether such effects might influence the relative orientations of clustered genes during evolution is yet to be tested (37). It is clear, however, that template topology and transcription are intricately interrelated in eukaryotes as well as prokaryotes, and the DNA topoisomerases presumably fulfill a key role in this interplay.

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