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DNA supercoiling in vivo

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DNA topoisomerase mutants of *Escherichia coli* and *Saccharomyces cerevisiae* were used to study the topological state of intracellular DNA. In *E. coli*, it is shown that switching off the gene *topA* encoding DNA topoisomerase I leads to an increase in the degree of negative supercoiling of intracellular DNA and inhibition of the growth of the cells: a $d(pCpG)_{16} \cdot d(pCpG)_{16}$ sequence on a plasmid is also shown to flip from a right-handed B-helical structure to a left-handed Z-helical structure in vivo when *topA* is switched off. In *S. cerevisiae*, the topological state of intracellular DNA is little affected by the cellular levels of the topoisomerases.

1. Introduction

Because of the double-helix structure of DNA, a duplex DNA ring can be viewed as two topologically linked single-stranded rings. The order of linkage is described by a quantity α termed the linking number. If the molecule is flatly laid on a plane, the linking number is equal to the number of helical twists or the number of times each strand revolves around the helical axis of the molecule (for more rigorous definitions of the linking number, see refs. 1–4).

In vitro, for an average DNA sequence free of strain, 10.5 base-pairs (bp) make a full helical turn under physiological conditions [5–13]. Thus a 'relaxed' DNA ring N bp in size is expected to have a linking number α^0 equal to $N/10.5$. Operationally, a DNA is in the relaxed state under a specified set of experimental conditions if repeated breakage and rejoining of a strand or strands under these conditions do not alter its linking number.

If the linking number α of a DNA differs from that of the same DNA in its relaxed state, α^0, the molecule is under strain. This often results in the coiling of the helical molecule in space, and the DNA is said to be supercoiled. It is positively supercoiled if $\alpha > \alpha^0$, and negatively supercoiled if $\alpha < \alpha^0$. The extent of supercoiling is conveniently expressed by the specific linking difference σ defined by $\sigma = (\alpha - \alpha^0)/\alpha^0$, which is termed the superhelical density or the 'titratable' superhelical density in the older literature. In a supercoiled DNA, there are both torsional and flexural strains. Supercoiling of a DNA therefore has strong effects both on structural changes in a DNA and on interactions between DNA and other molecules [14–20].

So long as the strands of a duplex DNA ring remain intact, the linking number α is a topological invariant, i.e., it is unaltered by environmental

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changes or deformation of the molecule. Therefore, the value α of an intracellular DNA ring can be measured after its isolation and purification; the measured value of α would in turn provide information on the state of the DNA in vivo. It is important, however, to prevent any interruption of the continuity of the DNA strands, even transiently, during the isolation of the DNA.

The degree of supercoiling of intracellular DNA in various organisms has been reviewed recently [21]. The values of σ for DNA rings isolated from both eubacteria and eukaryotes are typically around -0.06 where α^0 is taken to be the value of the relaxed form of the purified DNAs [16]. Because of interactions between intracellular DNA and components in the cellular milieu, however, there is some uncertainty in defining the relaxed state of intracellular DNA. For DNAs in eukaryotic cells, the negative values of σ can be attributed almost entirely to nucleosome formation [22] which lowers the linking number of a relaxed DNA relative to that of the same DNA when relaxed in its pure form [23]. DNAs inside eubacteria are negatively supercoiled [24]. The binding of cellular components also appears to reduce the linking number of the relaxed DNA, however, and the effective degrees of supercoiling of intracellular DNA in eubacteria are probably a factor of 2–3 lower than the specific linking differences with respect to pure DNA in its relaxed form as the reference state [25–27].

2. DNA supercoiling in eubacteria

In eubacteria, the degree of DNA supercoiling is dependent on the cellular levels of DNA topoisomerases (for reviews, see refs. 21, 25, 28 and 29). DNA gyrase (topoisomerase II) catalyzes the negative supercoiling of DNA in vivo as well as in vitro, whereas DNA topoisomerase I modulates the degree of supercoiling by relaxing negatively supercoiled DNA. In addition to biochemical evidence, the roles of the topoisomerases in the regulation of the degree of supercoiling of DNA in vivo are also suggested by the finding that *Escherichia coli* DNA topoisomerase I mutants are inviable unless compensated by other mutations, some of which are in DNA gyrase [30,31].

For two reasons, we undertook the construction of *E. coli* strains in which the gene *topA* encoding DNA topoisomerase I is expressed from a regulated *lac* promoter. First, placing the gene under the control of a promoter that can be turned on or shut off is one way to avoid the acquisition of compensatory mutations in a strain: the promoter is normally induced, and is repressed only during experimentation. Second, if the degree of supercoiling of intracellular DNA can be manipulated, then the effects of supercoiling on DNA structure and various cellular processes can be better studied. The construction of the *E. coli* strain 2034 *ΔtopA* pJW313 (*amp*^{R}P*lac-topA*) and its derivatives was outlined previously [32] and is described in more detail in footnote a of table 1. In these strains, the chromosomal copy of *topA* had been deleted and the *topA* is expressed instead from a *lac* promoter on a single-copy plasmid pJW313 derived from pDF41 [33], also described in the legend to table 1.

Assays of DNA topoisomerase I activity in lysates of the strains and the *topA*$^+$ parent strain *E. coli* 2034 carrying no plasmid indicate that *ΔtopA* strains carrying pJW313 (P*lac-topA*) and the *topA*$^+$ parent have about the same concentration of DNA topoisomerase I in the presence of isopropyl β-D-thiogalactopyranoside (IPTG), an inducer of the *lac* repressor which represses transcription from the *lac* promoter. In the absence of IPTG the level of the enzyme in *ΔtopA* (pJW313) strains is at most 5% of that of the *topA*$^+$ control (data not shown).

The functional importance of DNA topoisomerase I under normal physiological conditions is indicated in table 1. At 30°C, the growth rate of this strain in liquid medium is markedly affected by IPTG. Removal of IPTG causes a lengthening of cell doubling time by a factor of 1.5. Interestingly, at 42°C the growth rate is much less sensitive to the level of DNA topoisomerase I. The repression of the *topA* gene has an even more pronounced effect on growth rate if the intracellular level of *lac* repressor is increased by the introduction of a multicopy plasmid carrying a *lacI* gene with a promoter up-mutation. At a high level of *lac* repressor, growth rate is significantly re-

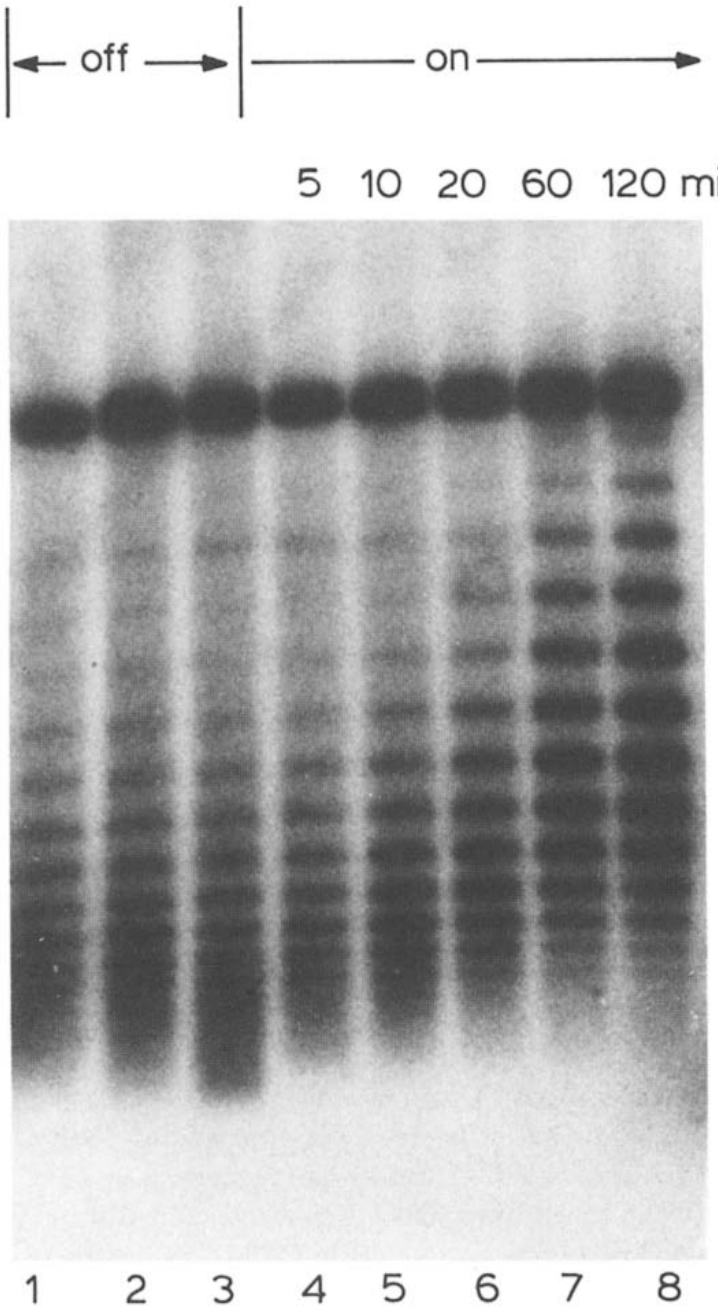

Fig. 1. The degree of supercoiling of intracellular DNA in *E. coli* is affected by the cellular level of DNA topoisomerase I. Cells carrying pACYC184 and a *topA* gene expressed from a *lac* promoter UV5 were grown at 42° C without IPTG overnight and then diluted and grown at 30° C for 2 h in the media described in footnote c to table 1, but with tetracycline (10 μg/ml) instead of kanamycin. The culture was divided in half, one part received IPTG to 80 μg/ml and aliquots were removed after 0, 60 and 120 min. Each was rapidly lysed upon removal by a procedure based on the method of Lockshon and Morris [39] in which each aliquot was immediately mixed with an equal volume of 2% SDS and 2 mM EDTA maintained at 65° C. Preparation of samples for gel electrophoresis and blot-hybridization for the analysis of topoisomer distributions of the plasmid pACYC184 were performed according to the procedures of Lockshon and Morris [39]. Electrophoresis was carried out in 1.1% agarose in Tris-borate-EDTA buffer containing 10 μg/ml of chloroquine. Lanes: (1–3) no IPTG, sampled at 0, 60 and 120 min, respectively; (4–8) with IPTG, sampled respectively at 5, 10, 20, 60 and 120 min following the addition of IPTG.

Table 1

Doubling time (in min) of *E. coli* strains [a] in which the *topA* gene is expressed from a *lac*UV5 promoter

lac repressor level	IPTG [b]	Doubling time [c]	
		30° C	42° C
Normal [d]	+	125	95
Normal	–	190	105
High [e]	+	120	80
High	–	270	140

[a] The parent strain *E. coli* 2034 *leu trp*E from the Yale stock collection was transformed with pJW313 (*amp*^{R} P*lac topA*), derived from a single-copy plasmid pDF41 [33] by replacing the *Sal*I to *Hin*dIII region of the plasmid with a 4.7 kb segment containing *amp*^{R} and the entire coding sequence of *topA* expressed from a *lac*UV5 promoter. A transformant was grown in the presence of IPTG, and P1 phage obtained from a strain DM700 (ΔCysB Δ*topA trp*E$^+$) was used to transduce the cells (for a physical map of this region, see ref. 62). Trp$^+$ Cys$^-$ transductants were selected, and the expected deletion of *topA* in the *E. coli* chromosome was confirmed by assays of extracts of cells grown in the presence and absence of IPTG. A *rec*A derivative of a transductant was constructed by transducing with phage P1 from W3110 Srl$^-$(Tn10)*rec*A (kindly provided by N. Kleckner) and selecting for tetracycline resistance. This *rec*A derivative was used in the experiments.

[b] Unless stated otherwise, IPTG was present at 80 μg/ml; all strains in which *topA* is expressed from *lac*UV5 were propagated in the presence of IPTG.

[c] Cell cultures were started from single colonies on LB plus IPTG plates and grown overnight at 42° C without shaking in M9 medium supplemented with 0.2% casamino acids, 50 μg/ml cysteine, 10 μg/ml thiamine, 20 μg/ml ampicillin, and 30 μg/ml kanamycin. They were then diluted 1 : 5 into several flasks in a gyratory shaker for growth measurements at 30 and 42° C with and without IPTG.

[d,e] To increase the repressor level, a 1100 bp fragment containing the entire *lac*I gene with an I^Q promoter up-mutation was cloned into the *Eco*RI site of pMK16 [33], which carries resistance to kanamycin. The plasmid was used to transform cells to *kan*^{R} and a high level of *lac* repressor; cells expressing the normal level of *lac* repressor were transformed with pMK16 without the *lac*I^Q insert. The 1100 bp *lac*I^Q fragment was constructed from pMC7 [63]; the boundary close to the promoter was originally defined by partial *Alu*I digestion to cut at a site approx. 250 bp upstream of a *Hin*c2 site; the other boundary was defined by Bal31 resection from a *Pst*I site to remove about 600 bp of sequence adjacent to the 3′-side of the gene.

duced even at the higher temperature (see data in table 1). When cells are grown on agar plates at 37° C, a large reduction in colony size is also observed in the absence of IPTG.

For ease in comparing the degrees of supercoiling under various conditions, a P*lac-topA* strain was first transformed with a small plasmid pACYC184 carrying resistance to tetracycline [34]. To reduce the chance of acquiring compensatory mutations, cells harboring pACYC184 were propagated in the presence of IPTG, and then grown overnight without IPTG at 42°C. The cells were diluted 1:20 into fresh medium at 30°C and allowed to equilibrate with shaking for 2 h before the culture was halved and IPTG was added to one culture. Samples taken after various times were quickly lysed as described in the legend to fig. 1. Crude preparations of DNA from the samples were loaded on an agarose gel. After electrophoresis in the presence of chloroquine [35] and transfer of the DNA to a nitrocellulose sheet according to the method of Southern [36], the sheet was hybridized with radioactively labeled pACYC184 to probe the pACYC184 DNA topoisomer distributions [37,38]. A comparison of samples from cells grown in the absence of IPTG (lanes 1–3) with samples on the right isolated after induction of the topoisomerases (lanes 4–8) shows that a significant relaxation of the plasmid becomes apparent as early as 10 min following induction of the gene. Inspection of the topoisomer patterns indicates a linking number increase of 5 ± 2 for this 4000 bp plasmid.

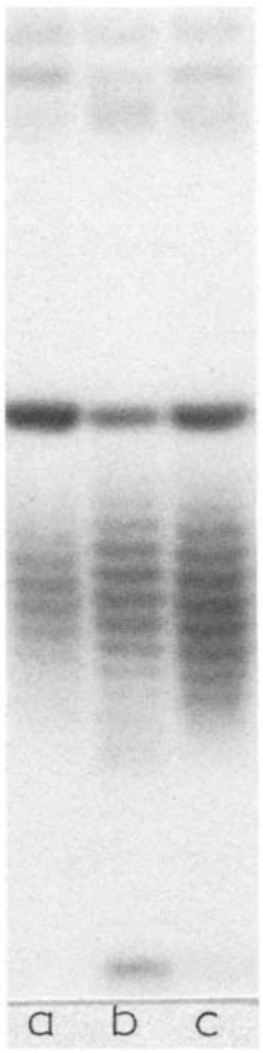

Fig. 2. Comparison of topoisomer distributions of 2-μm plasmids using three different lysis procedures. DBY745 (αadel–100 leu2–3 leu2–112 ura3–52) was grown at 30°C to $A_{595} = 0.5$ and then lysed. DNA was extracted and then electrophoresed in a 0.7% agarose gel in 0.2 M Tris-borate-0.2 M boric acid-0.004 M EDTA buffer containing 0.9 μg/ml chloroquine. The gel was then blotted [36] and hybridized with a radioactively labeled RNA probe [60] containing 2000 bp of the 2-μm plasmid sequence. Lanes: (a) glass bead lysis method [40]; (b) cold press lysis (see text); (c) zymolyase lysis [41].

3. DNA supercoiling in eukaryotes

In contrast to the results described above for DNA inside *E. coli*, the values of σ of plasmid DNAs isolated from the yeast *Saccharomyces cerevisiae* show little difference when the cellular levels of the DNA topoisomerases are varied by genetic and biochemical manipulations.

In *E. coli*, it is well known that the measured linking numbers of intracellular plasmids can be affected by the method of cell lysis used [39]. For this reason and because of the added difficulty in yeast of rapidly lysing cells, we isolated the endogenous 2-μm plasmid using three different lysis procedures and compared their topoisomer patterns. Lane a of fig. 2 shows a topoisomer distribution from a sample obtained using a glass bead lysis method [40]. The sample in lane c was obtained by using a standard zymolyase lysis method [41]. This procedure involves pelleting yeast cells, resuspension of the pellet in 1 M sorbitol-0.1 M EDTA-14 mM 2-mercaptoethanol, a lengthy incubation for 2 h with zymolyase at 37°C, digestion with proteinase K in 2% SDS, precipitation of the detergent with KCl, and finally phenol extraction and DNA precipitation. In lane b, the sample was obtained by a cell disruption procedure using an Edebo press [42]. Glycerol was rapidly mixed directly into a yeast culture to a final concentration of 10%, and the mixture was flash frozen by pouring it into liquid nitrogen. The frozen chunks of the culture were smashed with a

mallet and placed in the press which had been precooled to −70 °C. When the temperature of the press increased to about −30 °C, pressure was applied to force the frozen culture through a small orifice, causing cell disruption due to a phase transition of the ice. The disrupted cells were thawed by heating at 70 °C for 15 min in the presence of 1/10 vol. of 10% SDS, and the remaining steps in DNA preparation were the same as those used in the zymolyase procedure following the zymolyase treatment.

The cold press cell disruption and glass bead disruption procedure were designed to minimize the chance of topoisomerase actions during lysis, whereas the zymolyase procedure was not. Nevertheless, all three methods gave topoisomer patterns that are not significantly different (see fig. 2). There are two plausible interpretations for this lack of difference in the topoisomer distributions. Either there is negligible enzymatic activity using these lysis methods, or the intracellular plasmid DNA in vivo is in a relaxed state. If the DNA is relaxed, different degrees of enzymatic action would not affect the topoisomer distribution. We did observe, however, that the zymolyase and glass bead procedures consistently yielded more nicked DNA than the cold press method; thus, we favor the latter interpretation that the intracellular DNA is in a relaxed form.

A more direct test of the notion that the intracellular DNA is relaxed in yeast was performed by varying the levels of the topoisomerases inside the cell. In contrast to the case in *E. coli*, increasing or decreasing the cellular level of a topo-

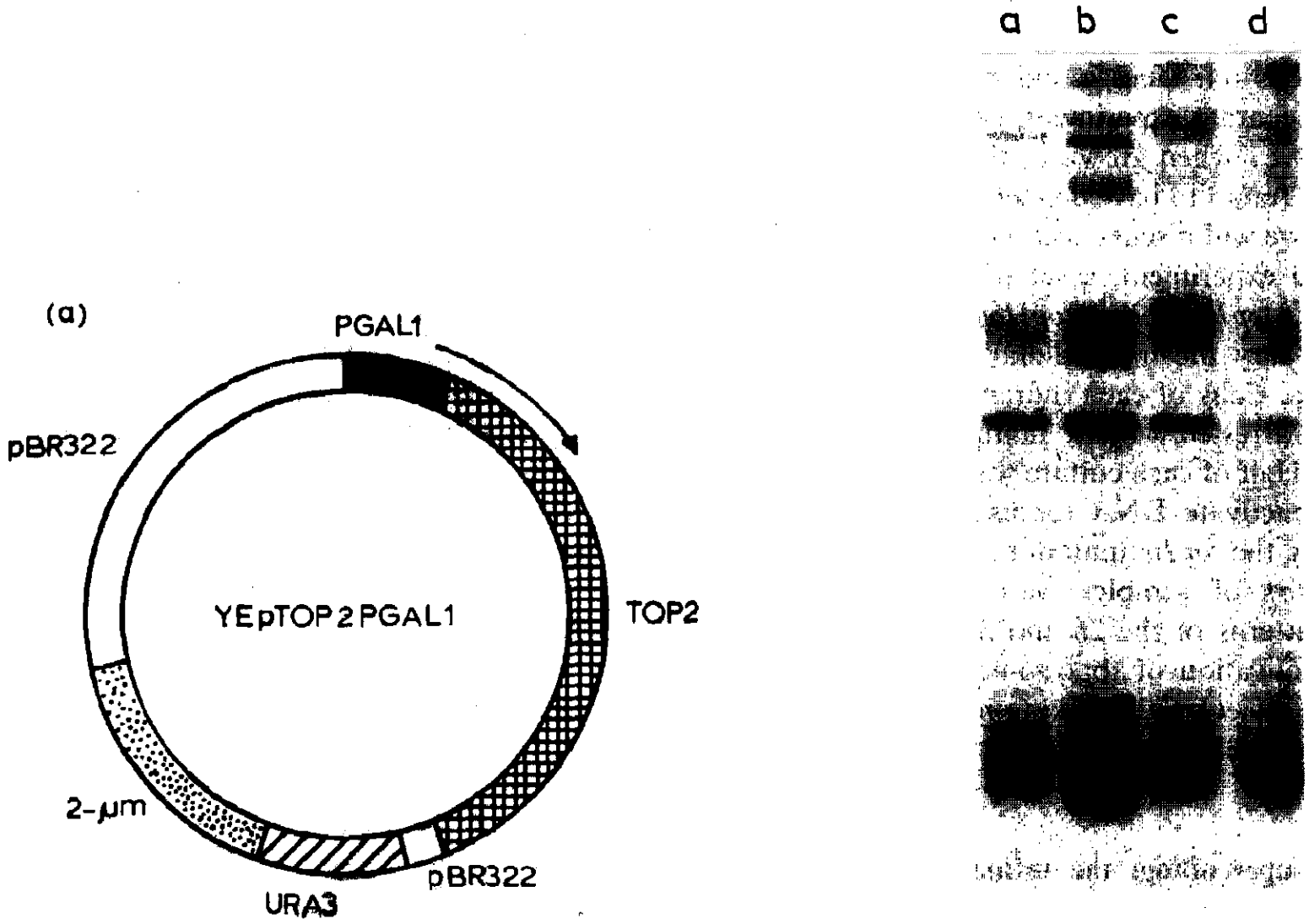

Fig. 3. (a). Plasmid YEpTOP2PGAL1. The entire coding sequence of yeast DNA topoisomerase II in between a *Bam*HI and *Ava*I site of pIIE101 [61] was placed downstream from a regulated promoter PGAL1. The promoter and the rest of the vector were derived from pCGS112 [43] and YEp24, respectively. (b) Degree of supercoiling of 2-μm DNA is not affected by the level of intracellular topoisomerase II in *S. cerevisiae*. Cells were grown in 2% lactate-3% glycerol media to $A_{595} = 0.6$. Glucose or galactose was then added to a final concentration of 2% and induction was for 13 h. Electrophoresis and Southern blot analysis were performed as described in fig. 2. Lanes: (a) untransformed DBY745 in glucose medium; (b) untransformed strain DBY745 in galactose medium; (c) DBY745 transformed with plasmid YEpTOP2PGAL1 in glucose medium; (d) DBY745 transformed with plasmid YEpTOP2PGAL1 grown in galactose medium.

isomerase in yeast shows little effect on the linking number distribution of a plasmid. In one experiment, yeast topoisomerase II was overproduced by transforming a strain with a plasmid, YEpTOP2PGAL1 (fig. 3a), which expresses yeast topoisomerase II from the galactose-inducible promoter GAL1 [43]. When this transformed strain is induced with galactose, active topoisomerase II protein is found to be overproduced at least 100-fold (S. Worland and J.C. Wang, unpublished data). Fig. 3b shows the linking number distributions of the 2-μm plasmid in *S. cerevisiae* strain DBY745 grown in the presence of glucose (lane a), or galactose (lane b). Lanes c and d show the linking number distributions of the 2-μm plasmid in the transformed strain grown in glucose and galactose medium, respectively. The topoisomer patterns show little difference among themselves or between them and samples isolated from untransformed cells (cf. lanes c and d with lanes a and b). In a separate experiment, cells harboring YEpTOP2PGAL1 when grown in the presence of galactose were found to have deleterious effects on growth which we will discuss elsewhere.

In a second experiment, a set of *TOP1TOP2*, *TOP1top2ts*, *Δtop1TOP2*, *Δtop1top2ts* mutants were used to vary the intracellular levels of the topoisomerases. Cells of each mutant were grown at 26°C and arrested with α mating factor for several hours. Half of each culture was then shifted to 35°C to inactivate DNA topoisomerase II in strains carrying the *top2ts* mutation, and 1 h afterwards the pairs of samples were lysed. DNA topoisomer patterns of the 26 and 35°C samples show that inactivation of topoisomerase I, II, or both has little effect on the linking number of the 2-μm plasmid (results not shown).

4. Localized supercoiling: the influence of transcription

The results presented above are concerned with the *average* degrees of supercoiling. In each case, the entire DNA ring forms a topological domain and the degree of supercoiling refers to the time average of this domain. Recently, it has been suggested that the transcription process might generate positively and negatively supercoiled loops locally [4,26,44]. If the positively and negatively supercoiled loops are relaxed at similar rates by the topoisomerases, they cancel each other and do not contribute to the average value. When the loops are relaxed differentially, however, they may contribute significantly to the average, and in some cases become the dominant factors [44]. The conditions which lead to the formation of such supercoiled loops have been summarized in the original work, and will not be repeated here.

The possibility of localized supercoiling by a protein tracking along a DNA [21,26,44,45] opens up an entirely new area of study on supercoiling-induced DNA structural changes in vivo and on their biological consequences.

5. Supercoiling induced structural transitions of DNA sequences in vivo

It is well known that supercoiling can have profound effects on structural transitions in a DNA [14,15]. In vitro, the formation of a pair of hairpinned structures (a 'cruciform') from a palindromic sequence, for example, occurs when σ is around -0.03 to -0.04 [46–50]. In vivo, examination of palindromic sequences yielded negative results [46,47,50,51]. There are two nonexclusive interpretations of the negative results. It is plausible that the effective degree of supercoiling in vivo is insufficient to drive cruciform formation; alternatively, the kinetic barrier may be too high for the formation of the cruciform. Recently, however, cruciform formation in vivo was implicated for a palindromic sequence in the plasmid colE1 [64].

We have tested the feasibility of manipulating the structure of a DNA sequence in vivo by switching on or off the *topA* gene. The transition of an alternating CG sequence from the right-handed B DNA structure to the left-handed Z DNA structure under the influence of DNA supercoiling [53] was examined. Several studies indicate that in wild-type *E. coli*, an alternating CG sequence exists as the B-helical form under normal growth conditions [55,56] (M. Gellert and G. Felsenfeld, personal communication). The results

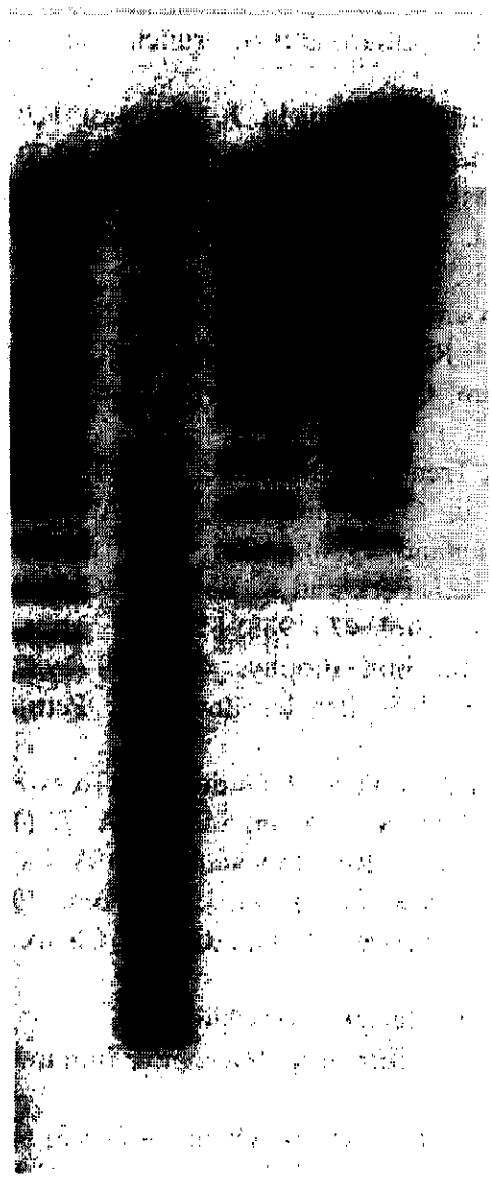

Fig. 4. Topoisomer distributions of pMK16-*lac*I^Q and pJW303 [pMK16-*lac*I^Q(CG)$_{16}$] insert in pJW303 can be in either the B-helical or Z-helical form inside *E. coli* depending on the cellular level of DNA topoisomerase I. Cells of the RecA$^+$ Δ*topA* pJW313 (*amp*^{R}P*lac-topA*) strain harboring pMK16 or pJW303 were grown at 42°C overnight without IPTG in M9 + 1% casamino acids + cysteine (50 μg/ml) with antibiotics as before. They were diluted into fresh media at 42°C with and without IPTG. After 1 h an aliquot of each culture was rapidly lysed by adding to an equal volume of 0.2 M NaOH, 1% SDS and vortex-mixed at room temperature. A calculated amount of 1 M HCl was added to neutralize the solution and DNA was isolated by alcohol precipitation following the removal of the detergent by precipitation with KCl. Gel electrophoresis was carried out in the same way as described in the legend to fig. 1. Lanes: (1) pMK16-*lac*I^Q, no IPTG; (2) pJW303 [*lac*I^Q(CG)$_{16}$], no IPTG; (3) pMK16-*lac*^{Q}, with IPTG; (4) pJW303, with IPTG.

shown in fig. 4 suggest, however, that by reducing the cellular level of DNA topoisomerase I the sequence can be driven into the left-handed Z form.

A pair of plasmids, pMK16-*lac*I^Q and pJW303 [pMK16-*lac*I^Q(CG)$_{16}$], were used to transform separately P*lac-topA* cells in which *topA* is expressed from a *lac*UV5 promoter. pMK16*lac*I^Q, which carries *kan*^{R} and overproduces *lac* repressor, is described in the legend to table 1. A 193 bp *Bam*HI fragment containing 16 CG units, derived from pLP417 [56], was cloned into the *Bam*HI site of pMK16-*lac*I^Q to give pJW313 [pMK16-*lac*I^Q(CG)$_{16}$].

In the presence of IPTG, the pair of plasmids show comparable degrees of supercoiling (fig. 4, lanes 3 and 4). Thus, with normal amounts of topoisomerase the sequence remains in the B conformation. In the absence of IPTG, however, the (CG)$_{16}$-containing plasmid shows a ladder of more negatively supercoiled topoisomers (cf. patterns in lanes 1 and 2 of fig. 4). We believe that this ladder is due at least in part to the flipping of the (CG)$_{16}$ insert to the Z-helical form in vivo. During electrophoresis in the presence of chloroquine, the alternating CG insert resumes the B-helical form, and the plasmid becomes more negatively supercoiled due to its lower linking number.

In the above experiments and in earlier studies of cruciform formation in vivo, no attempt was made to vary the positions of the supercoiling-sensitive structural elements relative to the locations of the transcription units. In view of the importance of localized supercoiling by transcription, a more systematic examination of supercoiling-induced structural transitions in vivo is needed.

6. Concluding remarks

In the sections above, we have presented our results on the state of intracellular DNA in *E. coli* and in *S. cerevisiae*. In accordance with the results of others [30,31,40,52,57,58], we show that switching off the *E. coli topA* gene increases the degree of negative supercoiling of intracellular DNA, whereas the linking number of intracellular DNA in the eukaryote *S. cerevisiae* is little affected by the cellular levels of DNA topoisomerases. These results are consistent with the notion that *on the average*, intracellular DNA is negatively supercoiled in eubacteria and is essentially relaxed

in eukaryotes. We have also presented evidence that by manipulating the cellular levels of DNA topoisomerases, structural transitions in DNA can be affected inside *E. coli*.

Recent analysis has pointed to the importance of localized supercoiling by proteins translocating along a DNA [45], and in particular by the transcription process [4,26,44]. Because of the high degree of supercoiling that can be achieved by such processes [52,59], the range in σ that can drive DNA structural transitions in vivo may be much larger than previously believed. A corollary of localized supercoiling is that the accessibility of regions of DNA to DNA topoisomerases may have profound effects on the supercoiling of these regions. Thus, DNA structural changes, DNA supercoiling, DNA topoisomerases and processes involving proteins moving along DNA are all intricately connected inside a cell. Many experiments can now be designed to elucidate these relations.

Acknowledgements

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