

Large-scale Effects of Transcriptional DNA Supercoiling *in Vivo*

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The scale of negative DNA supercoiling generated by transcription in Top⁺ *Escherichia coli* cells was assessed from the efficiency of cruciform formation upstream of a regulated promoter. An increase in negative supercoiling upon promoter induction led to cruciform formation, which was quantitatively measured by chemical probing of intracellular DNA. By placing a cruciform-forming sequence at varying distances from the promoter, we found that the half-dissociation length of transcription supercoiling wave is ≈ 800 bp. This is the first proof that transcription can affect DNA structure on such a remarkably large scale *in vivo*. Moreover, cooperative binding of the *ci* repressor to the upstream promoter DNA did not preclude efficient diffusion of transcriptional supercoiling. Finally, our plasmids appeared to contain discrete domains of DNA supercoiling, defined by the features and relative orientation of different promoters.

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Introduction

Transcriptional DNA supercoiling, postulated more than a decade ago by Liu & Wang (1987), attracted wide attention as a novel way of regulating local DNA structure and function (reviewed by Wang & Lynch, 1993; Droge, 1994). It originates from the basics of transcription: RNA polymerase unwinds a DNA segment at a transcription start site and translocates it along the transcribed gene (reviewed by Uptain *et al.*, 1997). This translocation forces DNA to rotate around the elongating RNA polymerase so that negative and positive waves of supercoiling are generated upstream and downstream of it, respectively (Liu & Wang, 1987; Wu *et al.*, 1988).

The existence of transcriptional DNA supercoiling *in vitro* has been convincingly demonstrated in several studies (Tsao *et al.*, 1989; Ostrander *et al.*, 1990; Droge & Nordheim, 1991; Droge, 1993). At the same time, a large body of data pointed out that purified RNA polymerases very efficiently transcribe circular DNAs (reviewed by

Chamberlin, 1974). This led to an early realization that torsional diffusion along DNA must be fast, so that the two supercoiling waves rapidly dissipate from the promoter and eventually cancel each other (Liu & Wang, 1987). If torsional diffusion *in vitro* is much faster than the transcription rate, one should expect only a subtle torsional gradient along each supercoiling wave. This was recently confirmed by estimating the distribution of a transcriptionally driven negative superhelical wave based on the efficiency of resolvase reaction (Wang & Droge, 1997).

Several additional elements could influence transcriptional supercoiling *in vivo*. The most important are DNA topoisomerases associated with transcription complexes (Gocke *et al.*, 1983; Fleischman *et al.*, 1984; Muller *et al.*, 1985; Gilmour *et al.*, 1986; Gilmour & Elgin, 1987; Ness *et al.*, 1988; Rose *et al.*, 1988; Zhang *et al.*, 1988; Stewart *et al.*, 1990) which can reduce transcriptional supercoiling or even cancel it altogether (Cook *et al.*, 1992). Second, since any natural DNA contains more than one promoter, simultaneous transcription from two neighboring promoters leads to either sequestration of positive and negative supercoiling domains or their mutual cancellation, depending on the relative orientation of the promoters (Wu *et al.*, 1988). Third, numerous factors, including transcription/

Abbreviations used: CAA, chloroacetaldehyde; IPTG, isopropyl β -D-thiogalactoside; DMS, dimethyl sulfate.

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translation coupling (Lodge *et al.*, 1989), membrane anchoring (Lodge *et al.*, 1989), large protein complexes assembled at structural elements of chromosomes (Gartenberg & Wang, 1993; Mirabella & Gartenberg, 1997), and protein-stabilized DNA loops (Wu & Liu, 1991), might affect the free rotation of transcribed DNA.

Thus, one would expect transcriptional supercoiling to be most prominent in cells with compromised DNA topoisomerases on DNA templates containing divergent promoters and other rotational barriers. Indeed, plasmids carrying divergent promoters become hyper negatively or positively supercoiled upon selective inactivation of topoisomerase I or DNA gyrase, respectively (Pruss & Drlica, 1986; Brill & Sternglanz, 1988; Wu *et al.*, 1988; Jaworski *et al.*, 1989). This effect was additionally enhanced (up to absolute superhelical density 0.1) by the presence of a rotational barrier caused by the coupled transcription-translation-membrane anchoring of a Tet-protein (Lodge *et al.*, 1989; Cook *et al.*, 1992; Lynch & Wang, 1993; Bowater *et al.*, 1994a,b; Mojica & Higgins, 1996).

More evidence for transcriptional supercoiling in Top⁻-cells came from the studies of the *Salmonella typhimurium leu500* mutation. This is an A-to-G transition in the TATA box of the leucine operon promoter which inactivates transcription (Mukai & Margolin, 1963). Deletion of the *topA* gene reactivates the *leu500* promoter (Trucksis *et al.*, 1981; Margolin *et al.*, 1985). This is not due, however, to an elevated level of global DNA supercoiling in TopI⁻ cells (Richardson *et al.*, 1984, 1988), but rather to a sequential activation of the two adjacent promoters, *ilvH* and *leuO*. It is believed that a negative supercoiling wave caused by the *ilvH* transcription first activates *leuO* transcription which, in turn, boosts *leu500* (promoter relay mechanism) (Wu *et al.*, 1995; Fang & Wu, 1998a,b). A cloned *leu500* promoter can also be activated by several other promoters *in cis* (Chen *et al.*, 1992; Spirito & Bossi, 1996).

Can transcriptional supercoiling subsist in normal cells with active DNA topoisomerases? This question was addressed by studying the formation of non-B-DNA structures as sensors for initiating DNA supercoiling (Rahmouni & Wells, 1989). A transient increase in negative supercoiling upstream of a promoter should promote the formation of these structures by suitable DNA sequences which can be detected by chemical probing of intracellular DNA. Using this approach, formation of cruciforms, Z-DNA, or H-DNA upstream of regulated promoters was shown in exponentially growing Top⁺ *Escherichia coli* cells (Dayn *et al.*, 1992; Rahmouni & Wells, 1992; Kohwi & Panchenko, 1993; Albert *et al.*, 1996). More sophisticated approaches detected Z-DNA upstream of transcribed genes in permeabilized mammalian cells (Wittig *et al.*, 1992; Wolfl *et al.*, 1996). Though there are some differences between those studies, several common conclusions could be drawn: (i) transcription modestly increases DNA supercoiling

upstream of promoters in Top⁺ cells (up to $|\sigma| \approx 0.05$); (ii) one promoter, rather than two divergent promoters, is necessary and sufficient for this increase; and (iii) transcription/translation coupling and membrane anchoring have no apparent effect on the supercoiling rate in Top⁺ cells. The interpretation of these data is that Topo I cannot completely relax negative supercoils progressively generated by RNA polymerase, leading to an increased supercoiling density upstream of promoters.

The scale of transcriptional supercoiling *in vivo*, however, is still unclear, i.e. the distance from the promoter at which transcriptional supercoiling is still pertinent. *A priori*, this could be any DNA length, since it depends on three unknown parameters: (i) the speed of torsional diffusion in DNA in the intracellular environment; (ii) the efficiency of topoisomerase relaxation of transcriptional supercoiling; and (iii) the effects of proteins bound to DNA on its rotation. To address this, we inserted cruciform-forming inserts at varying distances from a transcription start site and measured the efficiency of cruciform formation upon transcriptional induction *in vivo*. Our results show that there is only a twofold decrease in the intensity of cruciform formation between the -50 and -863 positions of the insert. Cooperative binding of the cI repressor does not preclude diffusion of transcriptional supercoiling. Thus, the effects of transcription on DNA structure *in vivo* are remarkably large-scale.

Results

Transcription induces cruciform formation far upstream of the promoter

A basic plasmid used in this study is shown in Figure 1(a). The initial construct, pTrcTACat/-185 (4999 bp), contains a cruciform forming a d(A-T)₁₆ insert 185 bp upstream of the transcription start of the inducible *trc* promoter. This promoter is a hybrid between the *trp* and *lac* promoters and is down-regulated by the lactose repressor. The same plasmid overexpresses lactose repressor from the *lacI^q* gene. As a result, in the absence of isopropyl- β -D-thiogalactoside (IPTG) the *trc* promoter is virtually silent. Except for its *XhoI* site, this plasmid is nearly identical with the pTrcTACat plasmid studied by us previously (Dayn *et al.*, 1992). In plasmid pTrcTACat/-863 (5677 bp), a 674 bp fragment of the coding part of the *cat* gene was inserted into the unique *XhoI* site of the pTrcTACat/-185, consequently changing the distance between the *trc* promoter and the cruciform to 863 bp. Finally, in plasmid pTrcTACat/-50 (4846 bp), the d(A-T)₁₆ repeat was moved immediately upstream of the *trc* promoter (-50 position). Note that the sizes of all these plasmids differ by no more than 17%. Thus, the maximum difference in the density of transcriptionally caused DNA supercoiling between those plasmids caused solely

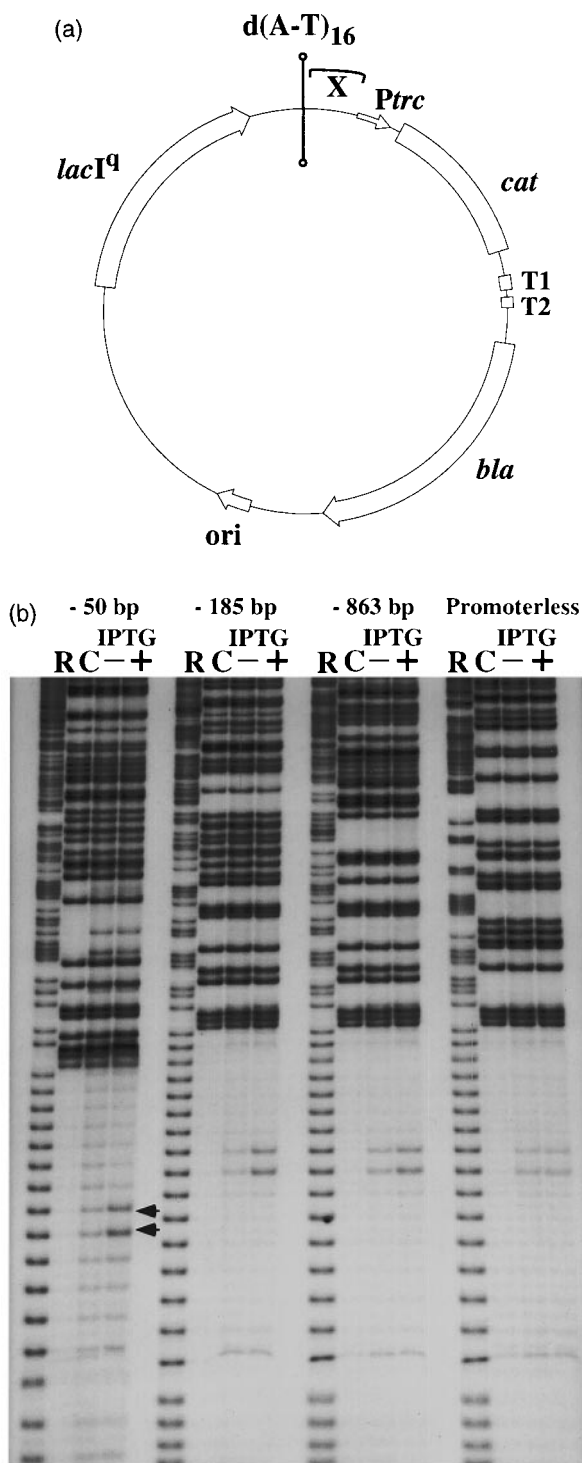


Figure 1. Cruciform formation at varying distances from the inducible promoter. (a) Schematic representation of the plasmids. X stands for the distance (50, 185, or 863 bp) between the d(A-T)₁₆ insert and *trc* transcription start site. (b) Detection of cruciforms by CAA-modification *in vivo*. The distance from the d(A-T)₁₆ insert to the *trc* start site is designated. The right-most panel corresponds to the plasmid without the *trc* promoter. R, C, Maxam-Gilbert sequencing reactions for unmodified DNA. IPTG + and – show C reactions for plasmid DNAs that were CAA-modified inside cells in the presence or absence of IPTG, respectively. Arrows indicate modified adenine bases.

by their size differences should not exceed this value. Also, the G + C-content of DNA sequences adjacent to the cruciform-forming insert is very similar for all these plasmids, varying between 50% and 56%. Thus, the kinetics of cruciform extrusion in all the cases should not be significantly affected by different flanking sequences.

Cells containing these plasmids were grown until early exponential phase in a specific medium: LB diluted threefold into a potassium, sodium phosphate (pH 7.0) buffer. Dilution of LB medium precludes leakage of the *trc* promoter without IPTG (data not shown). Buffering the medium is necessary to avoid pH changes during chloroacetaldehyde (CAA) modification. Cells were incubated with and without IPTG for ca one hour, followed by CAA modification. DNA samples were isolated and modified DNA bases were detected as described in Materials and Methods.

Figure 1(b) shows typical experimental data for the above plasmids. As one can see there is only marginal modification within the d(A-T)₁₆ insert of any construct in the absence of IPTG. This background level can be attributed to a small fraction of plasmid molecules containing cruciforms in the absence of transcription. Clearly, however, the addition of IPTG leads to the strong modification of two adenine bases in the middle of the d(A-T)₁₆ insert. This is exactly the modification pattern found in the case of cruciform formation by the d(A-T)₁₆ insert *in vitro* (Dayn *et al.*, 1991, 1992). Note also that for the pTACAT plasmid, lacking the *trc* promoter, modification within the d(A-T)₁₆ insert is at a background level. We conclude, therefore, that *trc* promoter activity leads to cruciform formation whether the d(A-T)₁₆ stretch is located 50 bp or 863 bp upstream of the transcription start.

The quantitative analysis of the results for several experiments is shown in Figure 2. The efficiency of cruciform formation appears to decrease exponentially with increasing distance between the cruciform-forming sequence and the promoter. However, the slope of this curve is very modest so that only a twofold decrease in cruciform formation is observed upon moving the cruciform-forming sequence 800 bp away from the promoter.

A question remains: what fraction of intracellular plasmid DNA contains cruciforms upon transcription induction? A direct answer could be given if we knew the efficiency of CAA modification of cruciform-containing DNAs in our *in vivo* conditions. Since this parameter is unknown, we have chosen a comparative approach based on the promotion of cruciform formation by chloramphenicol. Numerous studies (Haniford & Pulleyblank, 1983, 1985; McClellan *et al.*, 1990; Dayn *et al.*, 1991; Kohwi *et al.*, 1992) have previously shown that treatment of *E. coli* cells with an inhibitor of protein synthesis, chloramphenicol, leads to a substantial increase in the steady-state supercoiling of plasmid DNA, promoting the formation of non-B-DNA structures, including cruciforms. These structural transitions happen in a substantial fraction of

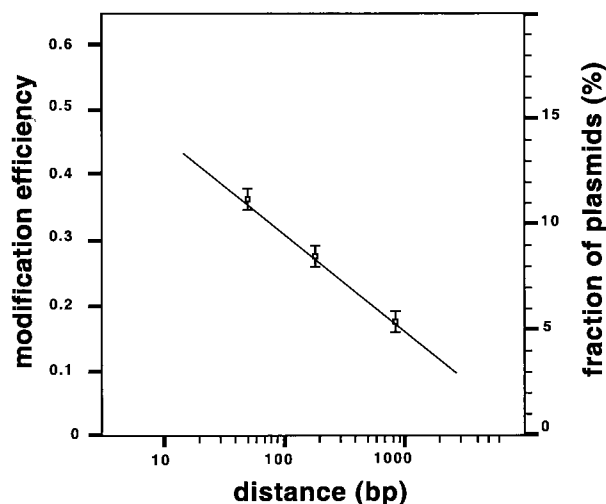


Figure 2. The dependence of cruciform formation efficiency on the distance from the *trc* promoter. Left vertical axis, normalized modification efficiency. Right vertical axis, the fraction of plasmid DNA containing cruciforms (see the text for details).

plasmid DNA and can be detected by topoisomer analysis. Comparing the fraction of plasmid DNA that underwent a structural transition (from topoisomer analysis) with the efficiency of CAA modification of the cruciform, one can estimate the relationship between the efficiency of cruciform formation and the fraction of plasmid DNA modified.

Figure 3(a) shows the topoisomer distributions for the pTrc-type plasmids with and without the d(A-T)₁₆ inserts isolated from cells treated and untreated with chloramphenicol. One can see that chloramphenicol increases the absolute negative supercoiling of control DNA, but much more so for the d(A-T)₁₆-containing DNA. Figure 3(b) shows the results of densitometric analysis of the topoisomer distribution. The lower panel shows the data for the control plasmid. It is obvious that the topoisomer distribution in the presence of chloramphenicol is shifted ca three supercoils in the negative direction relative to that in untreated cells. The topoisomer distribution in the presence of chloramphenicol has a single well-defined maximum and coincides with the theoretically expected distribution for a plasmid of this size (broken green line). The latter was calculated as $P = A \exp[-K\tau^2/N]$, where N is the plasmid length and $K = 1.1 \times 10^3$. The upper panel shows the data for the d(A-T)₁₆-containing plasmid. In the absence of chloramphenicol, the topoisomer distribution is practically identical with that of the control plasmid. In the presence of chloramphenicol, however, it is markedly different from the control situation: (i) it is much broader due to the existence of increased negatively supercoiled topoisomers; and (ii) it is bimodal. Our d(A-T)₁₆ repeat releases 3.5

supercoils during the cruciform formation (Vologodskaja & Vologodskii, 1999). Thus, if 100% of plasmids underwent cruciform formation *in vivo* under chloramphenicol treatment, one would expect topoisomer distribution to shift 3.5 negative supercoils compared to the control plasmid under the same conditions (shown by the broken orange line in the lower panel). Realistically, for the cruciform-containing plasmid we observe a bimodal distribution which is expected if only a portion of the plasmid population underwent cruciform transition *in vivo*. A theoretical topoisomer distribution calculated under the assumption that 56% of plasmids underwent this cruciform transition *in vivo* (red broken line in the upper panel) fits quite well with the experimental one. We conclude, therefore, that chloramphenicol induces cruciform formation in $\approx 56\%$ of plasmids with the d(A-T)₁₆ insert.

Figure 3(c) shows chemical modification of plasmid DNA from cells treated with chloramphenicol. One can see exactly the same pattern of modification as was seen above for transcription induction, reflecting cruciform formation. The rate of chemical modification is much more profound upon chloramphenicol treatment than after transcription induction. Hence, the modification efficiency of 1.8 corresponds to 56% of plasmids with cruciforms. Consequently, knowing the modification efficiency for the cruciform transition under the influence of transcription, we can calculate the fraction of plasmid DNA with cruciforms as 5 to 12%, depending on a distance of the repeat from the promoter (Figure 2 right vertical axis).

Protein binding to DNA upstream of the promoter does not affect cruciform formation

Considering the scale of the transcriptional DNA supercoiling *in vivo*, it is important to keep in mind that intracellular DNA is covered with proteins which may significantly affect torsional diffusion. In the above described experiments, the DNA separating the cruciform forming sequence and the *trc* promoter is "neutral", i.e. it is either the sequence upstream of *trc* promoter from the pTrc99 plasmid, or the coding portion of the *cat* gene. These sequences do not contain apparent sites for specific DNA-binding proteins.

In order to address the effects of a specific DNA-binding protein on transcriptional DNA supercoiling, we inserted two properly spaced operator sites, O_{R1} and O_{R2}, for the phage λ cI repressor (Ptashne, 1992) into the *XhoI* site of the pTrcTA-Cat/-185 plasmid. In the resultant plasmid, the d(A-T)₁₆ insert is located 234 bp upstream of the *trc* transcription start, but separated from the promoter by the cI operators situated around the -187 position. In order to look for the effect of cI binding on cruciform formation, we conducted experiments similar to those described above in two isogenic *E. coli* strains, AB313 and AB312, differing only by the presence or absence of lambda prophage, respectively. Thus, cI repressor is produced

in the AB313 strain, while the AB312 strain serves as a negative control. Figure 4(a) shows the results of the *in vivo* dimethyl sulfate (DMS) footprinting of the pTrcTACat/C1 plasmid in the two bacterial strains. Evidently, there is a methylation protection of the operator sites in the AB313 strain but not in the AB312 strain. Note also, that methylation protection is near complete and covers both operator sites. This implies that in the λ -lysogenic strain there is a strong cooperative binding of the cI repressor to most of the plasmid DNA molecules, notwithstanding its relatively high copy number.

Figure 4(b) shows the results for CAA modification of the d(A-T)_{16} insert in the two strains in the presence or absence of IPTG. There is no modification of the insert without transcription, but transcriptional induction leads to profound modification due to cruciform formation. It is obvious from Figure 4(b), that the efficiency of cruciform formation is nearly identical in both bacterial strains. We believe, therefore, that cooperative binding of the cI repressor to its two operator sites (i.e. the binding of four cI molecules of total molecular mass 104 kD) has very little, if any, effect on transcriptional DNA supercoiling.

Different domains of supercoiling within plasmid DNA

We show that in Top^+ *E. coli* cells, the level of DNA supercoiling upstream of the *trc* promoter is insufficient to cause tangible cruciform extrusion when transcription is off, but becomes sufficient for this upon transcription activation. From the structure of our plasmids (Figure 1), one can see that the d(A-T)_{16} repeat is located upstream of the inducible *trc* promoter but downstream of the constitutive *lacI^q* promoter. Thus, the actual level of DNA supercoiling in this area (and consequently the pattern of cruciform formation) may depend on three parameters: (i) the steady-state level of DNA supercoiling in the absence of transcription; (ii) the extra negative supercoiling generated by the *trc* transcription; and (iii) the extra positive supercoiling generated by *lacI^q* transcription. Could the pattern of cruciform formation change when the d(A-T)_{16} repeat is placed at different positions relative to different plasmid promoters?

In order to study this question, we constructed the plasmids presented in Figure 5(a). In pLacTA derivatives, either d(A-T)_{16} or d(A-T)_{11} repeats are situated upstream of the constitutive *lacI^q* promoter and downstream of the replication ori. Plasmid pTrcTAInvLac differs from the pTrcTACat/-185 by the inversion of the *lacI^q* gene. As the result, the d(A-T)_{16} insert is situated upstream of both *trc* and *lacI^q* promoters. The data on *in vivo* chemical probing of these plasmids are shown in Figure 5(b). First, one can see that the d(A-T)_{16} insert adopts cruciform conformation when it is located upstream of a single *lacI^q* promoter. The efficiency of CAA-modification in this case is very close to that observed under chloramphenicol treatment

(see Figure 3(b) for comparison): quantification of the results shows that cruciforms exist in approximately 58% of all plasmid molecules whether with or without IPTG.

Inversion of the *lacI^q* promoter in the pTrcTAInvLac plasmid leads to a similar modification pattern. The d(A-T)_{16} insert, which is now located upstream of both inducible and constitutive promoters, exists in a cruciform state in 50-60% of all plasmid molecules. There is no reliable increase in cruciform formation upon IPTG treatment.

Finally, the d(A-T)_{11} insert also forms cruciforms when upstream of the *lacI^q* promoter. CAA-modification in this case is less prominent relative to the d(A-T)_{16} repeat in the same location, but still quite evident. Quantification of the modification data shows that the d(A-T)_{11} repeat is in a cruciform state in $\approx 11\%$ of plasmid DNA molecules. At the same time, we were unable to detect any cruciform formation by the d(A-T)_{11} repeat upstream the *trc* promoter (data not shown).

We believe, therefore, that these data demonstrate the existence of different supercoiling domains in our plasmids, the borders of which are defined by transcription units. In the domain upstream of the *trc* promoter, the absolute level of negative supercoiling is relatively low and depends on promoter induction, while in the domain upstream of the *lacI^q* promoter, the level of negative supercoiling is constitutively high.

Discussion

Our data strongly indicate that transcription-generated DNA supercoiling is apparent even in *E. coli* cells with functional DNA topoisomerases. First, we demonstrate that induction of transcription increases the absolute level of negative supercoiling far upstream of the *trc* promoter. In fact, the half-dissociation length of the negative supercoiling wave generated by transcription *in vivo* appears to be roughly 800 bp. This is the first direct proof that transcription can affect DNA structure *in vivo* on such a large scale. Most of the previous estimates of this matter were based on an indirect approach (the rate of promoter activation at a distance), performed in Top^- cells, and gave contradictory results (Chen *et al.*, 1992; Tan *et al.*, 1994; Spirito & Bossi, 1996). The only such measurement in Top^+ cells was based on the efficiency of Z-DNA formation and gave results that were quite different from ours: increasing the distance between the two divergent promoters by an extra 800 bp precluded Z-DNA formation (Rahmouni & Wells, 1992). One explanation for this controversy could be that the 800 bp-long DNA segment chosen by Rahmouni & Wells (1992) not only mechanically increased the distance between the promoters but directly interfered with transcriptional DNA supercoiling because it contained barriers for DNA rotation, or hot-spots for topoisomerases, or because some DNA repeats

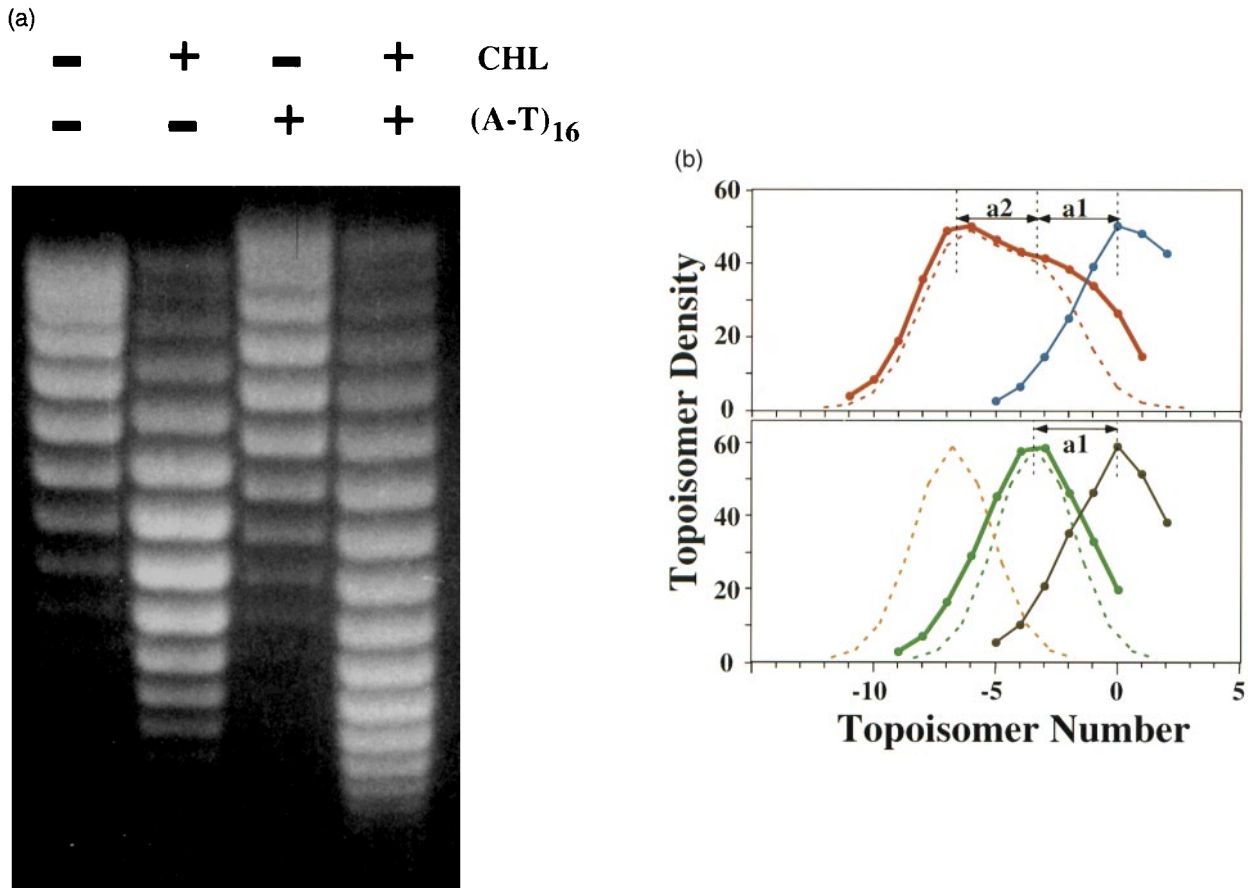


Figure 3 (Legend opposite)

adopt non-*B* conformations at lower superhelicity. Whatever the case, we never observed such effects with our intervening sequences that were of different length, origin and base composition.

Second, the level of negative supercoiling differs in different parts of our plasmids, which is likely determined by the features and relative orientations of plasmid promoters. We can roughly estimate the actual supercoiling density accumulated in the process of transcription. At physiological ionic strength *in vitro*, the d(A-T)₁₆ repeat adopts cruciform conformation at an absolute superhelical density of 0.05, while d(A-T)₁₁ requires at least 0.055 for this transition (Dayn *et al.*, 1991). Thus, absolute superhelical density upstream of the induced *trc* promoter should be around 0.05, but less than 0.055. At the same time, absolute superhelical density upstream of the *lacI^q* gene should constitutively be more or equal to 0.055. Previously, substantial differences in actual supercoiling densities between different plasmid segments were also observed by others (Rahmouni & Wells, 1989, 1992; Zheng *et al.*, 1991). The interpretation of those data, however, was somewhat complicated since the plasmids under investigation carried

either the *tet* gene, the expression of which anchors the plasmid changing both constrained and unconstrained supercoiling (Albert *et al.*, 1996; Chen *et al.*, 1998), or closely spaced divergent promoters. Our experimental system lacks these caveats, but the conclusion is the same regardless.

The fact that the absolute value of negative supercoiling increases substantially upon induction of a single promoter stands out for two reasons. Firstly, the calculations described in the Supplementary Material show that RNA polymerase should not generate any visible torsional stress on a naked circular DNA if it freely rotates around itself in water. In fact, for a 5 kb plasmid, the maximum induced supercoiling density (σ_{\max}) would be only $\sim 10^{-6}$ and its relaxation time (τ) is only ~ 20 μ s. Since we observe a detectable transcriptionally induced torsional stress *in vivo*, rotation of DNA inside the cell must be hindered by higher viscosity of the intracellular medium, the presence of multiple DNA-binding proteins and/or DNA kinks. It may not be that surprising in this regard that additional binding of a relatively small cI repressor does not affect transcriptional DNA supercoiling.

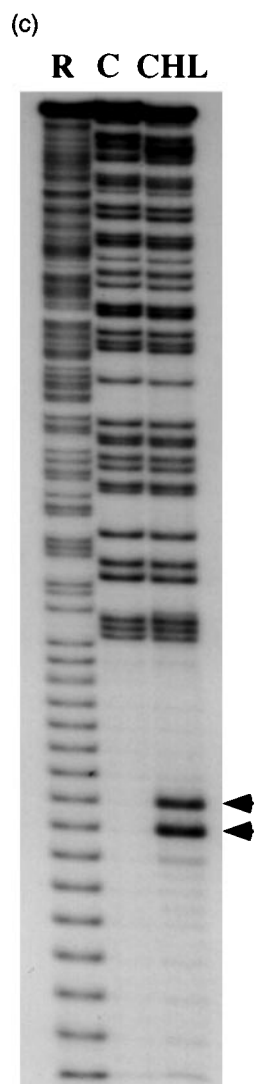


Figure 3. The effect of chloramphenicol on cruciform formation. (a) Topoisomer distribution for plasmids with and without a d(A-T)₁₆ insert in the presence or absence of chloramphenicol (CHL). (b) Densitometric analysis of topoisomer distribution. Lower panel: Control plasmid. Brown line, untreated cells; green line, chloramphenicol-treated cells; broken green line, theoretical distribution for the control plasmid from chloramphenicol-treated cells; broken orange line, theoretical distribution for d(A-T)₁₆-containing plasmid from chloramphenicol-treated cells assuming that all the plasmids underwent cruciform transition. Upper panel: Cruciform-containing plasmid. Blue line, untreated cells; red line, chloramphenicol-treated cells; broken red line, theoretical distribution for chloramphenicol-treated cells assuming that 56% of the plasmids underwent cruciform transition. a1, shift in topoisomer distribution caused by chloramphenicol; a2, shift in topoisomer distribution caused by cruciform formation. (c) Detection of cruciforms by CAA-modification *in vivo*. R, C, Maxam-Gilbert sequencing reactions for unmodified DNA. CHL, C reaction for plasmid DNA which was CAA-modified in chloramphenicol-treated cells. Arrows indicate modified adenine bases.

Secondly, we do not see substantial differences in the level of supercoiling in DNA segments situated between the two divergent promoters (*lacI^q* and *trc*) or upstream of a single *lacI^q* promoter in Top⁺ cells (Figure 5(b)). A potential concern here is that the *trc* promoter is ca fivefold weaker than the *lacI^q* promoter with regard to cruciform extrusion *in vivo*, so that its contribution may be insignificant. In our previous study, however, we did not see much difference between a single or divergent *trc* promoters (Dayn *et al.*, 1992). This refutes data that in Top⁻ cells the superhelical density between divergent promoters is immensely greater than that upstream of a single promoter (Wu *et al.*, 1988; Jaworski *et al.*, 1989; Chen *et al.*, 1993). This discord can be explained assuming that only a small fraction of the transcriptionally generated torsional stress in Top⁺ cells escapes relaxation by topoisomerases and rapidly distributes along the plasmid DNA.

We show that the efficiency of cruciform formation, i.e. the level of negative supercoiling, upstream of the promoter was not affected by binding of the cI repressor to its operator sites located between the cruciform and promoter. A potential problem here is whether cI binding in our plasmids *in vivo* is complete. The binding constant of the repressor dimer to the O_R1 site is 3×10^{-9} M and this binding additionally improves by a factor of 2 due to the cooperativity between the two operator sites. At the same time, the concentration of the cI repressor in a λ lysogen is maintained at $\approx 4 \times 10^{-7}$ (reviewed by Ptashne, 1992). This means that practically all the operator sites in our case should be occupied by the repressor. Consistently, we see a very strong protection of plasmid operator sites from DMS-methylation *in vivo* (Figure 4(a)). Another potential concern lies in the on-off rate of the cI-DNA complex. One may argue that if the dissipation rate of the supercoiling wave is comparable with the dissociation rate of

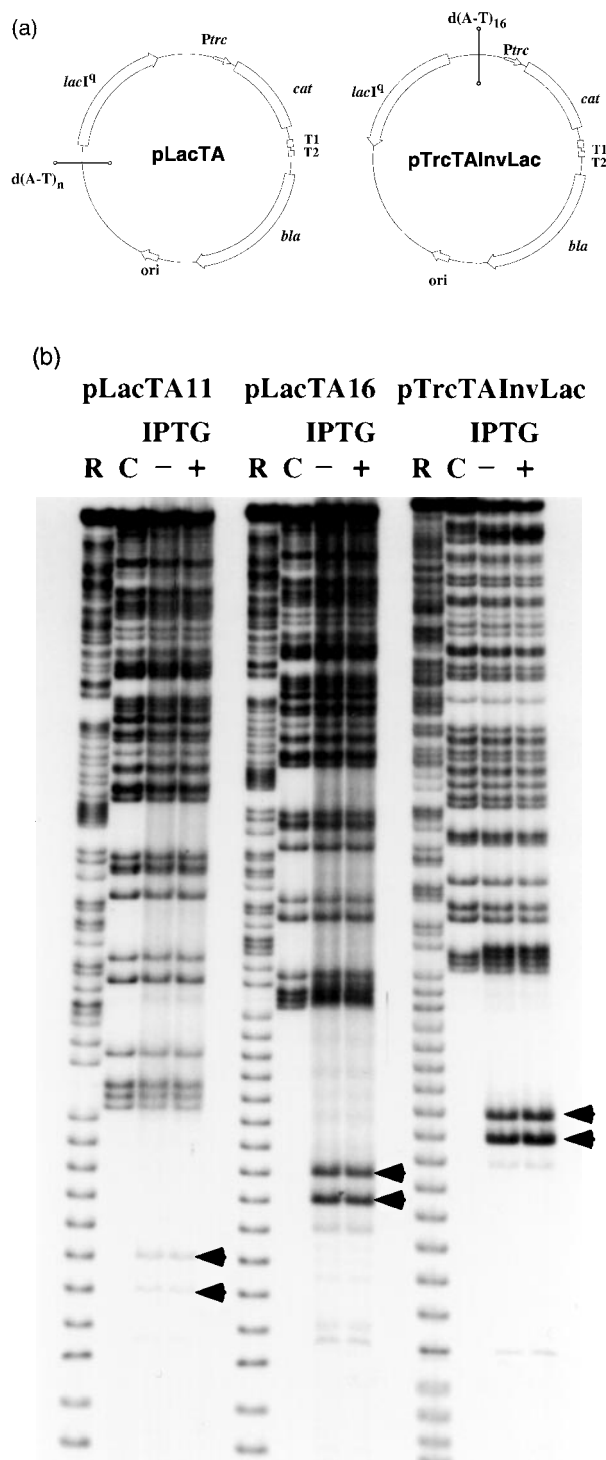


Figure 5. Cruciform formation in different domains of plasmid DNA. (a) Schematic representation of the plasmids. (b) Detection of cruciforms by CAA-modification *in vivo*. Plasmids are designated above the sequencing ladders. R, C, Maxam-Gilbert sequencing reactions for unmodified DNA. IPTG + and - show C reactions for plasmid DNAs that were CAA-modified inside cells in the presence or absence of IPTG, respectively. Arrows indicate modified adenine bases.

Plasmid pTACat was obtained by deleting a 242 bp *KpnI-KpnI* fragment, containing the *trc* promoter, from the pTrcTACat/-185 plasmid.

Plasmid pTrcCat was obtained from pTrcTACat/-185 by removing a 59 bp *PstI-KpnI* fragment, containing the (AT)₁₆ stretch.

Plasmids pLacTA16 and pLacTA11 were obtained by inserting annealed oligonucleotides Lac116/Lac216 or Lac111/Lac211, correspondingly, into the *NsiI* site of the plasmid pTrcCat.

Plasmids were primarily maintained in *E. coli* strain XL1-Blue (Stratagene). For the experiments with the C1 repressor, a pair of isogenic strains AB312 (Hfr13 *thr-1*, *leuB6*, *lacZ4*, *glnV44(AS)*, *rpsL8*, *thi-1*, λ^-) and its λ^+ -derivative AB313 (kindly provided by Dr Mary Berlin from the *E. coli* Stock Center) were used.

Plasmid DNA was isolated by an alkali lysis protocol, followed by equilibrium centrifugation in a CsCl/EtBr gradient (Sambrook *et al.*, 1989).

Chemical probing of intracellular DNA

An overnight bacterial culture was diluted 100-fold in 100 ml of fresh LB/3 broth (3.33 g/l Tryptone, 1.67 g/l yeast extract, 3.67 g/l NaCl, 4.6 g/l Na₂HPO₄, 2 g/l KH₂PO₄, 0.67 g/l NH₄Cl, 0.4% (w/v) glucose) containing 100 μ g/ml of ampicillin and grown at 37°C with vigorous aeration to $A_{600} = 0.2-0.3$. IPTG (Sigma) was added up to 2 mM, where required, and the culture was grown until $A_{600} = 0.7-0.8$ followed by chemical modification.

For chloramphenicol experiments, cells were grown in the medium to $A_{600} = 0.4$, chloramphenicol was added up to 170 μ g/ml, and cells were further incubated for four hours followed by chemical modification.

For cruciform detection, chloroacetaldehyde (CAA) (Fluka) was added to the cell culture up to 4%. After 20 minutes incubation at 37°C with constant shaking, 500 ml of warm (37°C) LB/3 broth was added. After a further five minutes incubation with constant shaking, cells were abruptly cooled to $\approx 0^\circ\text{C}$ by the addition of 300 ml of ice saturated with 160 mM NaCl, then centrifuged, washed with ice-cold TES buffer, and plasmid DNA was immediately isolated.

For dimethyl sulfate (DMS) footprinting, cells were incubated with 0.025% DMS for five minutes at 37°C, cooled, centrifuged, washed with TES buffer, followed by isolation of plasmid DNA.

Mapping of modified DNA bases

Approximately 0.2 μ g of plasmid DNA was digested with corresponding restriction enzymes, labeled with [³²P]dCTP using the Klenow fragment of DNA polymerase I, followed by the isolation of a 3'-end-labeled fragment from the 5% PAAG. In DMS experiments, end-labeled DNA fragments were cleaved with 20% piperidine (Fisher) for 30 minutes at 100°C. For CAA experiments, end-labeled DNA fragments were treated with hydrazine in high salt for seven minutes at 25°C (C reaction) followed by cleavage with piperidine. The products of cleavage were separated on a 10% sequencing gel followed by autoradiography and quantitative analysis.

The following end-labeled DNA fragments were used in Maxam-Gilbert sequencing: the 277 bp *XbaI-EcoRI* fragments of either pTrcTACat/-185 or pTrcTAInvLac; the 326 bp *XbaI-EcoRI* fragment of the pTrcTACat/C1;

the 191 bp *XbaI-ScaI* fragment of the pTrcTACat/-863; the 303 bp *XbaI-EcoRI* fragment of the pTACat; the 265 bp *Clal-AluI* fragment of the pLacTA16; the 255 bp *Clal-AluI* fragment of the pLacTA11; the 452 bp *BfmI-EcoRV* fragment of the pTrcTACat/-50; and the 196 bp *TaqI-EcoRI* fragment of the pTrcTA11Cat/-50.

Quantitative analysis of the intensity of chemical modification was carried out on the 445 SI Phospho-Imager (Molecular Dynamics). The intensity of band corresponding to modified DNA base was normalized to the intensity of band, corresponding to regular C reaction on the same line.

Electrophoretic analysis of DNA topoisomers

Cells were grown as described above. After four hours incubation with 170 µg/ml of chloramphenicol, plasmid DNA was isolated by an alkali lysis protocol, treated with RNase A followed by triple deproteinization by phenol, phenol/chloroform and reprecipitation with ethanol. Gel electrophoresis was performed at room temperature in 1% agarose gel in the presence of 6 µg/ml of chloroquine (Reanal) with constant recirculation of the Tris-acetate buffer at 2.5 V/cm for 16 hours followed by ethidium bromide staining as described (Dayn *et al.*, 1991). Densitometric analysis of topoisomer distributions was carried out with an Eagle Eye II system (Stratagene).

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