*Proc. Natl. Acad. Sci. USA*
Vol. 87, pp. 8373–8377, November 1990
Biochemistry

# Superhelical torsion in cellular DNA responds directly to environmental and genetic factors

(DNA supercoiling/cellular DNA topology/cruciform/topoisomerase/osmium tetroxide)

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*Communicated by I. Tinoco, Jr., July 13, 1990*

**ABSTRACT** **Superhelical tension of DNA in living bacteria is believed to be partially constrained by interaction with proteins. Yet DNA topology is a significant factor in a number of genetic functions and is apparently affected by both genetic and environmental influences. We have employed a technique that allows us to estimate the level of unconstrained superhelical tension inside the cell. We study the formation of cruciform structures by alternating adenine-thymine sequences in plasmid DNA by *in situ* chemical probing. This structural transition is driven by superhelical torsion in the DNA and thus reports directly on the level of such tension in the cellular DNA. We observe that the effect of osmotic shock is an elevation of superhelical tension; quantitative comparison with changes in plasmid linking number indicates that the alteration in DNA topology is all unconstrained. We also show that the synthesis of defective topoisomerase leads to increased superhelical tension in plasmid DNA. These experiments demonstrate that the effect of environmental and genetic influences is felt directly at the level of torsional stress in the cellular DNA.**

DNA supercoiling is thought to play a significant role in a number of genetic processes in prokaryotes, and yet the nature and extent of superhelical torsion present in the living cell are incompletely understood at present. When DNA such as a circular plasmid is extracted from cells, it is found typically to have a linking deficiency equivalent to about 1 turn in 20. However, the linkage deficiency exhibited in the isolated DNA may not be manifested as unconstrained torsion in the DNA when it is in the living cell, and a number of estimates suggest that the real value of the effective level of supercoiling *in vivo* is about half that of the purified DNA (1–5). Thus a significant fraction of the linkage deficiency may be absorbed by wrapping about cellular proteins. Determination of linking difference of isolated DNA may be a valuable indication of the *in vivo* state of the DNA, yet this may reveal only part of the real situation.

DNA supercoiling is thought to respond to a number of cellular processes. Measurement of plasmid linking differences has indicated that supercoiling may be influenced by environmental factors, such as anaerobiosis (6) and osmolarity (7). It is clearly also under the direct influence of topoisomerase enzymes (8–10), including topoisomerase I and DNA gyrase. Most recently, it has been shown that local superhelical stress can be influenced by the act of transcription (11–15). Since the strength of a number of bacterial promoters depends on the level of DNA supercoiling (16), it is clear that genetic function and DNA topology may be linked at a number of levels of complexity.

One way in which to gauge the level of local superhelical torsion in DNA is to introduce a sequence that can undergo a topology-dependent structural isomerization. A number of kinds of DNA sequences can undergo a profound alteration in structure, that is dependent both on the local DNA sequence and on the level of DNA supercoiling. For example, the formation of both left-handed Z-DNA by $(C\text{-}G)_n$·$(C\text{-}G)_n$ sequences (17) and cruciform structures by inverted repeats (18–20) is energetically favored by negative supercoiling, and therefore such sequences may be used to report on local superhelical torsion. To do this, it must be possible to detect the DNA structural transition *in situ* inside the cell.

Three methods have been employed to reveal the formation of altered DNA structures in cells. First, their existence may be indicated by certain genetic consequences, including a propensity to undergo deletion of inverted repeats (3, 21, 22) and potential Z-forming sequences (23), and modulation of transcription by cruciforms (24) and Z-DNA (2). Comparison of the susceptibility of sequences of various size to exhibit such effects with the known *in vitro* properties of the sequences enables an estimate to be made of the effective (i.e., unconstrained) superhelix density inside the cell. For example, by comparing the genetic stabilities of a series of alternating adenine-thymine sequences $[(A\text{-}T)_n]$ (3), we were able to estimate the effective superhelical density as −0.025 in *Escherichia coli*, a value that is in excellent agreement with that deduced by a totally independent approach based on the topology of the integration of phage λ (4). A second method reveals structural transitions in DNA by virtue of alterations in the topology of the DNA (25, 26); cellular topoisomerases compensate for relaxation due to local conformation transitions by altering the linking number. Zacharias *et al.* (5) have observed bimodal topoisomer distributions in plasmids bearing inserts that can form Z-DNA, from which they have deduced a superhelix density of −0.025. In addition, this method has been employed to explore cruciform formation by $(A\text{-}T)_n$ sequences in cells exposed to chloramphenicol (26).

Perhaps the most direct way in which to demonstrate formation of a perturbed structure in the cell is by probing the DNA *in situ*. This can be achieved by using an enzyme, whose synthesis must be induced in the cell, or a chemical probe. Inhibition of methylation has been used to demonstrate Z-DNA formation (27), and cleavage by a four-way junction resolvase has been used to reveal cruciform formation (28). Chemicals have been used very successfully to probe DNA structure (29–37), and these may be directed to cellular targets provided they can be introduced into the cell. We have shown (38–40) that osmium tetroxide·2,2′-bipyridine may enter bacterial cells, where it may react with bases present at a potential B–Z junction, suggesting the formation of Z-DNA in these cells. Recently, Rahmouni and Wells (41) have used *in situ* chemical modification of B–Z junctions in *E. coli* to demonstrate variation in supercoiling between different loci in the plasmid pBR322.

Osmium tetroxide reacts selectively with the unpaired thymine bases of cruciform loops, and the resulting adduct

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may be cleaved using piperidine. We chose to examine cells transformed with a series of plasmids bearing $(A\text{-}T)_n$ inserts, since these are known to form cruciforms *in vitro* with relatively low free energies of formation and, rather importantly, no kinetic barriers (1, 26, 42–44). We show that we can detect cruciform extrusion *in situ* in cells that have undergone salt shock, and we can use the probing results to assess the effects of environmental and genetic factors on the supercoiling of cellular DNA in a rather direct way.

## MATERIALS AND METHODS

**Plasmids.** The plasmids used in these studies were all derivatives of pXG540(1), containing an $(A\text{-}T)_{34}$ sequence or a shorter tract derived from a perfect deletion of a number of A-T dinucleotides. *E. coli* HB101 transformed by the required plasmid was grown in M9 salts minimum medium, with amplification for 14–16 hr by chloramphenicol (150 μg/ml). DNA was isolated by lysis in lysozyme, SDS, and EDTA and purified by two rounds of cesium chloride density gradient centrifugation in the presence of ethidium bromide. Supercoiled plasmid recovered by side puncture was extracted with cold 1-butanol and dialyzed against 10 mM Tris·HCl (pH 7.5) and 0.1 mM EDTA at 7°C.

***In Situ* Chemical Modification.** *E. coli* transformed with pXG540 was grown with shaking at 37°C to an $OD_{550}$ of 0.5 in 50 ml of supplemented M9 salts. Chloramphenicol (150 μg/ml) was added, and the culture was shaken for 15 hr at 37°C. Osmotically shocked cells were obtained by adding NaCl to the culture to a concentration of 0.5 M and incubating at room temperature for 40 min. Cells were collected by centrifugation and washed with 100 mM phosphate (pH 7.2). DNA was modified *in situ* by incubating cells in 200 mM phosphate (pH 7.2), 1 mM osmium tetroxide, and 1 mM bipyridine in a total volume of 10 ml for 20 min at room temperature. The reaction was stopped by washing cells in cold buffer. Plasmid DNA was prepared according to a modified procedure of Holmes and Quigley (45). Approximately 1-μg quantities of the modified DNA were digested with *Eco*RI and radioactively labeled by using [$\alpha$-$^{32}$P]dATP and DNA polymerase, and the full-length plasmid DNA was purified by electrophoresis in agarose. The DNA was subjected to incubation with 1 M piperidine at 90°C for 30 min to cleave osmate adducts, and analyzed by electrophoresis on a 7% polyacrylamide sequencing gel containing 7 M urea and autoradiography.

***In Situ* Modification in BR83.** *E. coli* K12 strain BR83 (*topA51$_{am}$ argA supD74$_{ts}$ rpsL Δlac-514*) was constructed by R. Depew and given to us by C. J. Dorman (46). HB101 (*topA*$^+$) and BR83 (*topA51$_{am}$ supD74$_{ts}$*), each transformed with pXG540, were grown at 30°C (permissive temperature) in supplemented M9 medium to a cell density corresponding to $OD_{600}$ of 0.8. The cultures were then incubated for 3 hr at 42°C (nonpermissive temperature), with or without prior addition of chloramphenicol. Just before *in situ* modification, chloramphenicol was added to those cultures that had not already received it. Osmium tetroxide modification was performed directly on the cultures by addition of 0.1 volume of 2 M phosphate buffer (pH 7.2), 20 mM osmium tetroxide, and 20 mM bipyridine and incubating at 42°C for 5 min. The reaction was stopped by washing the cells with ice-cold 100 mM phosphate buffer (pH 7.2). DNA was prepared and cleaved with piperidine as before, except that an additional restriction cleavage with *Hae* III after isolation of the *Eco*RI fragment was introduced. This cleaved the $^{32}$P-labeled *Eco*RI fragment into two radioactively labeled pieces, one of which was only 19 base pairs in length and was therefore too small to appear on the gel, thus reducing the background on the autoradiograph.

## RESULTS

**Formation of Cruciform Structures in Bacteria in Response to Salt Shock.** The plasmids employed in these studies were related to pXG540, which contains a section derived from a *Xenopus* αT1-globin gene, including a sequence of 34 uninterrupted repeats of ApT [i.e., $(A\text{-}T)_{34}$]. The alternating section forms a cruciform structure *in vitro* that is characterized by having a relatively low free energy of formation and the absence of a kinetic barrier to its extrusion (1). Thus in every respect, this sequence is an excellent candidate for cellular cruciform formation. It has been shown that transcriptional activity may affect local superhelix density (11–15) and that this may influence the stability of DNA segments that can undergo structural interconversion. However, there is no transcription through the $(A\text{-}T)_n$ tracts in these plasmids, and the promoters present are all relatively weak and constitutive.

*E. coli* HB101 cells transformed with pXG540 were grown to mid-exponential phase, followed by growth for a further period in the presence of chloramphenicol. The cells were then subjected to osmotic shock in 0.5 M sodium chloride. This procedure has been shown (7) to increase the superhelix density of plasmids isolated from cells, and the treatment changed the linking difference of extracted pXG540 DNA by about six turns (Fig. 1). After harvesting and resuspension, the cells were incubated with 1 mM osmium tetroxide and 1 mM bipyridine in phosphate buffer at room temperature. Plasmid DNA was isolated and digested with *Eco*RI, followed by radioactive labeling of 3′ termini by using DNA polymerase. The DNA was purified by isolation from an agarose gel after electrophoresis, and osmate adducts were cleaved by hot piperidine. The resulting fragments were examined by electrophoresis on a sequencing gel and autoradiography.

The results are shown in Fig. 1. Two points are firmly established. First, we can clearly detect cruciform formation inside the cell. The DNA from the salt-shocked cells has suffered a pronounced modification of thymine bases at the center of the $(A\text{-}T)_{34}$ tract, consistent with formation of a cruciform by the $(A\text{-}T)_n$ sequence. Second, the effect of the salt-shock treatment is clear. A significant extent of cruciform formation is observed *only* for the salt-shocked cells. Chloramphenicol treatment alone produced no discernible topological changes. This indicates that an elevated effective cellular superhelix density occurs in response to the salt shock, such that the $(A\text{-}T)_{34}$ cruciform becomes stable in the cellular plasmid.

**Cruciform Formation Depends on the Length of the $(AT)_n$ Tract: Estimation of Effective Cellular Supercoiling Following Salt Shock.** If the extent of cruciform formation reflects the level of unconstrained supercoiling inside the cell, then the use of a series of $(A\text{-}T)_n$ sequences should make calculation of the effective superhelix density possible. Since the length of the $(A\text{-}T)_n$ tract will determine the change in twist on cruciform formation, it will fix the critical level of supercoiling at which the cruciform becomes stable: the longer the $(A\text{-}T)_n$ tract, the lower the superhelix density that is required to stabilize the cruciform. In the course of previous studies of $(A\text{-}T)_n$ sequences, we have isolated a series of deletion mutants of pXG540 that are altered only in the length of the $(A\text{-}T)_n$ tracts themselves. This series of species was treated exactly as above, and the result is presented in Fig. 2. It is clear that the degree of modification is a function of the length of the $(A\text{-}T)_n$ tract in the plasmid, with the longer tracts undergoing the greatest extent of cruciform modification. $(A\text{-}T)_{34}$ and $(A\text{-}T)_{22}$ are equally well modified (and thus we refer to this as 100% relative modification), $(A\text{-}T)_{12}$ is completely unmodified, whereas $(A\text{-}T)_{15}$ shows an intermediate level of modification (30% maximum).

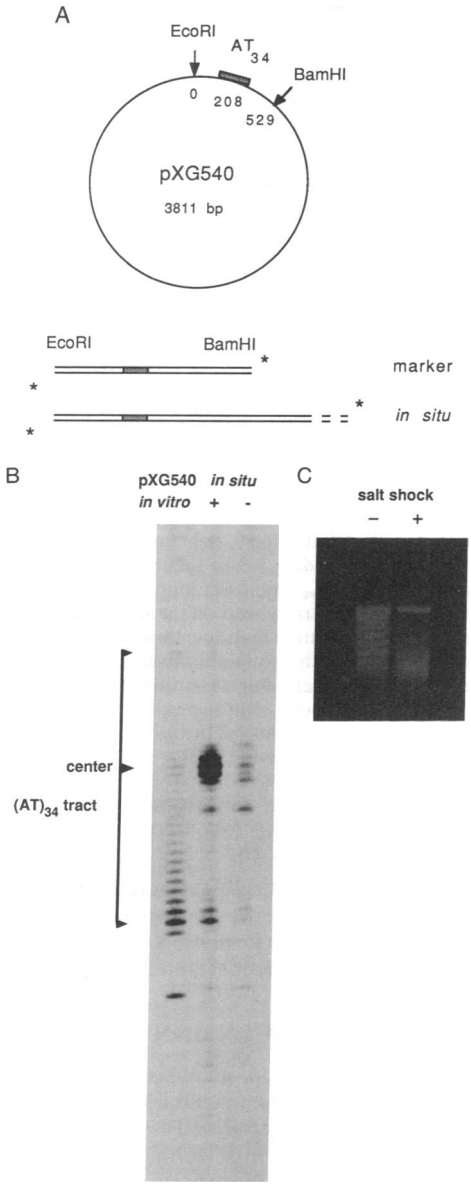


FIG. 1. Chemical modification of a cruciform structure adopted by $(A\text{-}T)_{34}$ sequence present in *E. coli*. (*A*) Map of pXG540 showing the $(A\text{-}T)_{34}$ sequence (stippled). In the *in situ* modification experiments, the plasmid was cleaved with *Eco*RI and radioactively labeled at the resulting 3′ termini. For *in vitro* modification of pXG540 as a marker, the DNA was cleaved with *Eco*RI and *Bam*HI and labeled at the resulting 3′ termini. (*B*) *In situ* modification of pXG540 with and without salt shock. The DNA in the left-most lane was obtained by *in vitro* modification of pXG540 with osmium tetroxide under conditions where there is uniform reactivity of all thymine bases in the $(A\text{-}T)_{34}$ tract (43) and can be used to identify the extent of the alternating sequence, indicated by the arrowheads on the left side. Note the strong modification at the center of the $(A\text{-}T)_{34}$ tract by osmium tetroxide, provided that the cells were subjected to the salt-shock procedure. The middle and right lanes show *in situ* modification with (+) and without (−) salt shock. (*C*) Effect of salt shock on the linking number of plasmid extracted from *E. coli*. Cells were grown as above, with and without salt shock, and pXG540 DNA was prepared. This was electrophoresed in a 1% agarose gel in 90 mM Tris·borate (pH 8.3), 10 mM EDTA, and chloroquine (4 μg/ml). Note that the salt-shocked DNA is more supercoiled than that of the untreated cells, by approximately six turns.

We conclude that these differences reflect different extents of cruciform extrusion by the various $(A\text{-}T)_n$ tracts inside the cell, and the data are quantified in Table 1. Since the free

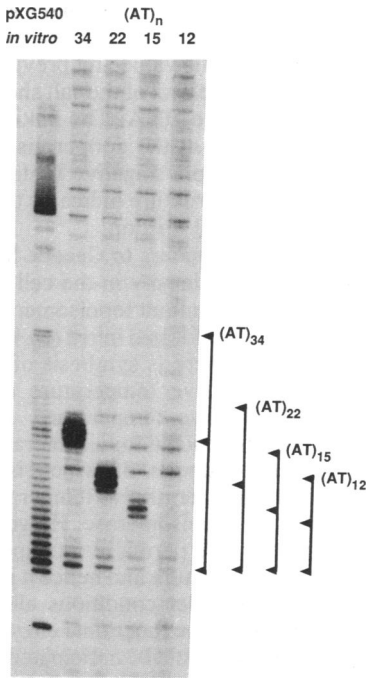


FIG. 2. Cellular cruciform formation by $(A\text{-}T)_n$ sequences as a function of inverted repeat length. *E. coli* cells transformed with different plasmids related to pXG540, but containing $(A\text{-}T)_n$ tracts of different lengths, were salt shocked and treated with osmium tetroxide exactly as before. The lane on the left shows uniform *in vitro* osmium tetroxide modification of pXG540 (as in Fig. 1, this was cleaved with *Eco*RI and *Bam*HI before labeling, and therefore the $(A\text{-}T)_{34}$ tract appears twice in this lane only, due to labeling at each end). The extent and centers of the $(A\text{-}T)_n$ sequences are denoted by the arrowheads shown on the right. The 5′ ends of each tract (lower end of the gel) are at equivalent positions in each case. The additional bands seen in all tracks of *in situ* modification arise from cleavage elsewhere in the molecule.

energy of formation of the $(A\text{-}T)_n$ cruciform is well known (1, 42), we can calculate the critical topoisomer linking difference required for cruciform formation for each plasmid. Any topoisomer more negatively supercoiled than this value will contain a stable $(A\text{-}T)_n$ cruciform. These values can be used to deduce the mean superhelix density of the plasmid DNA inside the cell, under the conditions of the experiment. A calculated Boltzmann distribution of topoisomers at 37°C can only be fitted to the observed relative extents of modification

Table 1. Correlation between the extent of chemical modification corresponding to cruciform formation and the superhelix density required to form a stable cruciform structure by $(A\text{-}T)_n$ sequences of different lengths

| $n$ | $\Delta Lk_{crit}$* | $\sigma_{crit}$† | % modification‡ | % modification (calculated)§ |
|---|---|---|---|---|
| 12 | −19 | −0.055 | 0 | 0 |
| 15 | −16 | −0.044 | 30 | 35 |
| 22 | −12 | −0.033 | 100 | 100 |
| 34 | −9.5 | −0.026 | 100 | 100 |

*Calculated critical linking difference ($\Delta Lk_{crit}$) at which the cruciform becomes stable in a 3.8-kilobase plasmid, based on a free energy of formation of 13 kcal·mol$^{-1}$.
†Critical superhelix density, calculated from $\sigma_{crit} = \Delta Lk_{crit}/Lk^\circ$, where $Lk^\circ$ is the relaxed linking number of the plasmid.
‡Relative extent of chemical modification of loop sequences estimated by densitometry.
§Calculated relative modification, assuming a Boltzmann topoisomer distribution (47–49) at 37°C with $\sigma = -0.041$ (see Fig. 3). This was calculated on the basis of full extrusion by all topoisomers more supercoiled than the critical linking difference.

of the different sequences in one way, giving a value of mean superhelix density of −0.041 for the salt-shocked cells (Fig. 3). The difference between this value and previous estimates of −0.025 shows directly the effect of the salt shock treatment on the *in vivo* topology of the DNA. The linking number of plasmid extracts from BR83 at the nonpermissive temperature was correspondingly more negative (data not shown); the formation of cruciform structure indicates that this is due to unconstrained superhelical tension.

**Cruciform Extrusion in Response to Genetic Mutation.** The level of effective superhelix density in the cell may be modulated genetically by using a mutant topoisomerase I. pXG540 [containing $(A\text{-}T)_{34}$] was transformed into a $topA51_{am}\ supD74_{ts}$ strain of *E. coli*, in which there is synthesis of normal topoisomerase I at the permissive temperature and defective enzyme at the nonpermissive temperature. In this strain (BR83), production of a functional topoisomerase I requires a suppressor tRNA, the synthesis of which is temperature sensitive. Growth at the nonpermissive temperature results in the synthesis of defective topoisomerase. However, the topoisomerase synthesized at the permissive temperature is still functional at elevated temperature and hence it is necessary to incubate for some hours under conditions allowing protein synthesis in order to replace the functional enzyme. Synthesis of defective topoisomerase would be anticipated to result in an increased level of cellular supercoiling, and we set out to test this by using the *in situ* cruciform formation assay.

*In situ* modification with osmium tetroxide was performed on BR83 cells containing $(A\text{-}T)_{34}$ plasmid, but in contrast to the experiments reported above, no salt shock was included. The results are presented in Fig. 4. Modification corresponding to cruciform formation is apparent following a shift to the nonpermissive temperature. An identical experiment using pXG540 transformed into the $topA^+$ HB101 resulted in no modification at the $(A\text{-}T)_n$ tract. Similarly, when chloramphenicol was added to prevent protein synthesis after the temperature shift, no cruciform extrusion was detected.

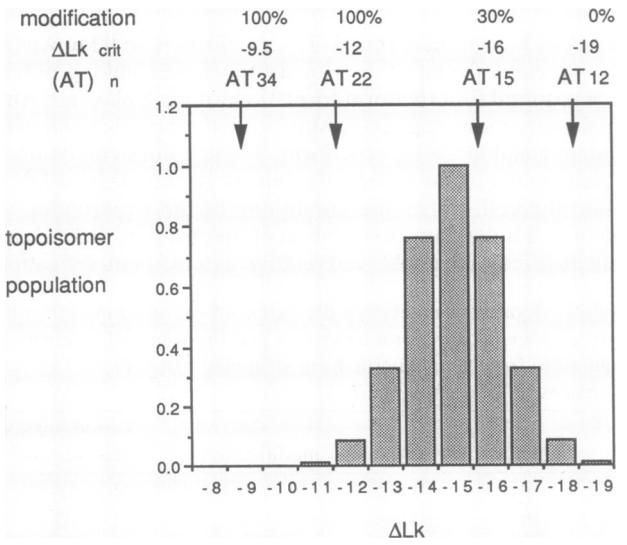


FIG. 3. A Boltzmann topoisomer distribution about $\Delta Lk = -15$ is consistent with the relative extents of chemical modifications of the $(A\text{-}T)_n$ sequences. Theoretical topoisomer populations were calculated as a function of mean linking difference and fitted to the calculated critical linking differences for cruciform formation by the different $(A\text{-}T)_n$ tracts. The expected extent of cruciform formation can be calculated on the assumption that all topoisomers more supercoiled (i.e., to the right of the arrow denoting the $\Delta Lk_{crit}$) will support cruciform formation and hence be chemically modified *in situ*. The profile shown is that giving the experimentally observed ratios of cruciform modification by the different plasmids and is centered on $\Delta Lk = -15$, corresponding to $\sigma = -0.041$.

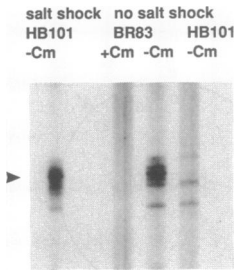


FIG. 4. A genetic lesion in topoisomerase I leads to increased cellular cruciform extrusion. The gel shows the result of an experiment in which cruciform extrusion was probed *in situ* in two strains of *E. coli* in which there was normal (HB101) and temperature-sensitive (BR83) synthesis of topoisomerase I respectively. BR83 ($topA51_{am}\ supD74_{ts}$) was grown at 30°C (permissive temperature) to a cell density corresponding to $OD_{600}$ of 0.8, followed by incubation for 3 hr at 42°C (nonpermissive temperature), with or without prior addition of chloramphenicol (Cm). Synthesis of the defective topoisomerase could be prevented by inclusion of chloramphenicol. In the control study with HB101 ($top^+$, right lane), the cells were treated identically to BR83, including a 3-hr incubation at 42°C. Chemical modification of the $(A\text{-}T)_{34}$ cruciform loop resulted in bands at the position indicated by the arrowhead on the left. Note that there was osmium tetroxide modification at the center of the $(A\text{-}T)_{34}$ tract in the topoisomerase mutant at the nonpermissive temperature, without a requirement for salt shock, but that this did not occur in the chloramphenicol treated cells or in the $top^+$ cells. For comparison, the left-most lane shows the result of *in situ* chemical modification of the $(A\text{-}T)_{34}$ tract in salt-shocked HB101.

Synthesis of the defective topoisomerase I results in an effective DNA supercoiling inside the cell such that the $(A\text{-}T)_{34}$ cruciform is now stable in the absence of salt shock. The linking number of pXG540 extracted from BR83 at the nonpermissive temperature was correspondingly more negative (data not shown); the formation of cruciform structure shows that this is due to unconstrained superhelical tension.

## DISCUSSION

We have exploited the *in situ* analysis of a structural transition in DNA to study torsion in cellular DNA. The formation of a cruciform inside bacterial cells is a direct response to superhelical stress. Using this technique, we have demonstrated that the physiological response of bacteria to certain environmental and genetic changes is an increased level of torsional stress in the cellular DNA.

Our chemical probing has shown that cruciforms can exist inside cells. This should not be taken to mean that they necessarily *do* exist in cells under normal conditions. In these experiments, we severely biased the system in favor of cruciform formation by two devices. First, we used either salt shock or a topoisomerase mutation to raise the superhelix density, and second we introduced $(A\text{-}T)_n$ sequences into the cells, that form cruciforms relatively easily. Nevertheless, the results indicate that there are conditions under which cruciform extrusion might occur.

We have exploited cruciform formation by the $(A\text{-}T)_n$ sequences to report on the effective superhelix density of plasmid DNA in *E. coli* under a variety of conditions. The information that comes from these experiments is different from that derived from measurements of linking number of DNA extracted from the cells, although it is complementary. It is not possible to interpret the linking difference of isolated DNA unambiguously in terms of torsional stress in the DNA in the cells, because not all of the linking deficiency may be unconstrained. A change in the linking number of extracted plasmid could be due to wrapping around proteins, with no change in torsional stress, for example. The use of the *in situ* detection of cruciform extrusion reveals that the effects of

salt shock and topoisomerase deficiency can be manifested directly in terms of torsional stress present in the cellular plasmid DNA.

Growth of enterobacteria in medium of high osmotic strength results in the elevation of DNA supercoiling in plasmids isolated from the cells (7). Salt or sucrose shock for brief periods may also alter the linking number of isolated plasmid DNA from *E. coli* HB101 (J.A.M., unpublished data). This change occurs rapidly, does not require protein synthesis, and is stable under some conditions even after transfer to medium of lower ionic strength. In the absence of salt shock, the effective level of superhelicity in pXG540 plasmid DNA is believed to be of the order of $-0.025$ (1, 3), deduced from the genetic stability of $(A\text{-}T)_n$ tracts, and this is increased to $-0.041$ by the salt treatment. We have shown here that the linking number change in the extracted plasmid is mirrored by a change in the torsional stress in the plasmid DNA inside the cell. Quantitative comparison of these results indicates that the increased supercoiling due to salt shock (six turns) is virtually all unconstrained. In terms of free energy of supercoiling, the effect of the salt shock is to increase the available energy of plasmid supercoiling by nearly 3-fold.

In addition to environmental factors, plasmid linking number may also be affected by genetic factors, notably by mutations in topoisomerase and gyrase genes (8–10). We have shown that once again these effects are paralleled by the direct measure of effective torsional stress *in situ*, measured by the effect on cruciform extrusion by the reporter $(A\text{-}T)_n$ sequence.

An additional factor that has been shown to affect the level of supercoiling in DNA is local transcriptional activity. Liu and Wang (12) first proposed that an elongating RNA polymerase with associated transcripts might be rotationally hindered, leading to domains of positive supercoiling ahead of, and negative supercoiling behind, the polymerase. The $(A\text{-}T)_n$ sequences studied here were located in regions not undergoing active transcription, and the results should therefore represent basal levels of effective DNA supercoiling under the particular conditions of the experiment. Rahmouni and Wells (41) have estimated that local superhelix density can vary over the range $-0.021$ to $-0.038$. The method of studying cruciform formation by $(A\text{-}T)_n$ sequences suggests itself as a powerful topological probe with which to investigate domains of supercoiling inside the cell.

We thank C. J. Dorman, N. Ni Bhriain, and C. F. Higgins for helpful discussion; the Medical Research Council, the Science and Engineering Research Council, and the Cancer Research Campaign for financial support; and the Royal Society and the Czechoslovak Academy of Sciences for a travel fellowship (to J.A.M.).

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